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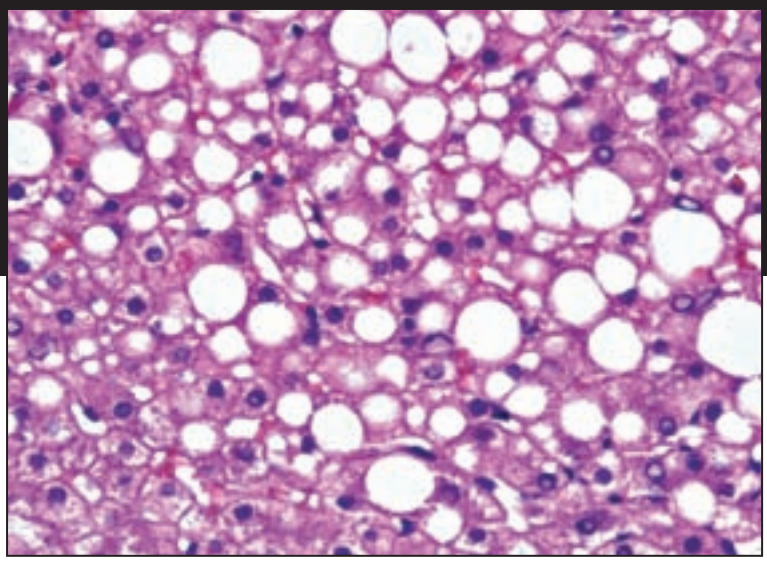
METABOLIC ASPECTS OF CHRONIC LIVER DISEASE



Ami Schattner
Hilla Knobler
Editors

NOVA

METABOLIC ASPECTS OF CHRONIC LIVER DISEASE

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AMI SCHATTNER
AND
HILLA KNOBLER
EDITORS

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PREFACE

'Metabolic aspects of chronic liver disease' is a subject that has been practically transformed in recent years. It reveals not only fascinating research achievements, but also their practical translation to the bedside and to improved patient care and better patient outcomes. All these are presented here by leaders in the field whose own research has made significant contributions to our understanding of the metabolic aspects of chronic liver diseases and is bound to do so in the future as well.

Nonalcoholic fatty liver disease (NAFLD) is now recognized as the most prevalent disorder of the liver in developed countries, related to obesity and insulin resistance. It comprises a spectrum of hepatic pathology from benign steatosis to the more severe form of nonalcoholic steatohepatitis (NASH), that can lead to the dreaded complications of cirrhosis and hepatocellular carcinoma. Three whole chapters are devoted to NAFLD due to its prevalence and public health burden on one side, and to the growing research and expanding knowledge on the other.

Keith Lindor and **Phunchai Charatcharoenwitthaya** from the Mayo Clinic at Rochester, provide an excellent review of the clinical and histological aspects of NAFLD. They summarize the epidemiological data, highlighting the rapid increase in NAFLD related to the epidemic of obesity and the metabolic syndrome. The most common presentation of NAFLD is asymptomatic increased liver enzymes but nonspecific clinical features may be associated. Various imaging modalities can diagnose liver steatosis and ultrasonography is the most widely used. However, liver biopsy may still be needed in some patients to confirm the diagnosis, exclude other etiologies and perform staging. The authors provide clinical parameters that can guide the clinicians in decisions regarding the performance of a liver biopsy and its use to determine patient care and long-term prognosis.

A comprehensive review by **Brent Neuschwander-Tetri** from Saint Louis University Missouri and **Metin Basaranoglu** from Selcuk University, Turkey, describes in great depth the pathophsiology of NAFLD and NASH. Genetic and environmental factors lead to insulin resistance and inflammation, both having pivotal role in NAFLD. Insulin resistance in adipose tissue leads to increased peripheral lipolysis and elevated non-esterified fatty acids. Accumulation of triglycerides in hepatocytes, the hallmark of NAFLD, is a result of an increased non-esterified fatty acids pool, due to increased uptake, increased de-novo synthesis, impaired intracellular catabolism and impaired secretion of triglycerides in the

form of very-low-density lipoproteins (VLDL). The progression of NAFLD to NASH may be the consequence of secondary abnormalities such as injured and dysfunctional mitochondria, generation of reactive oxygen species, lipid peroxidation, disturbed production of adipocytokines and gut-derived cytotoxic products. This event which occurs in a minority of patients is central to the development of the more severe complications.

Stephen Malnick from the Hebrew University Hadassah, Jerusalem, **Yitzhak Halperin** from Ashkelon, Israel and **Lee Kaplan** from the MGH, Boston, discuss the available therapeutic options in NAFLD - "an entity in evolution". They describe the difficulties arising from the lack of uniform diagnostic criteria and existence of different subsets of patients. In addition, many studies are small and non-randomized making informed decisions difficult. Nevertheless, they present interesting and important data demonstrating that weight loss is an effective treatment resulting in improved biochemical markers as well as histological findings. Other therapeutic modalities including insulin sensitizing agents, antioxidants and probiotics may also have a beneficial role, but the authors conclude that further studies are needed before they become part of the routine treatment of NAFLD.

Hilla Knobler and **Ami Schattner** of the Hebrew University Hadassah Medical School, Jerusalem, analyze the compelling evidence of the increased prevalence of type 2 diabetes mellitus among patients with chronic hepatitis C which was found to be striking even in the absence of liver cirrhosis. These relatively recent observations have already generated a unifying hypothesis that links liver inflammation and fibrosis with inflammatory cytokines, resulting in insulin resistance in susceptible patients. The practical implications of this important association affecting an enormous number of patients worldwide are discussed, as well as directions for future research.

The role of iron toxicity in other chronic liver diseases such as: alcoholic liver disease, NASH, chronic hepatitis C and porphyria cutanea tarda is discussed by **Bruce Bacon** from Saint Louis University School of Medicine, and by **John Olynyk, John Ombiga** and **Debbie Trinder** from Fremantle Hospital, Western Australia. In an intriguing review they provide data for the complex interaction between iron toxicity and the development of advanced hepatic fibrosis and cirrhosis in alcoholic liver disease, and poor response to interferon therapy in chronic hepatitis C.

David Lomas and **Meera Mallya** and **Russel Phillips** from the University of Cambridge, United Kingdom, discuss in unusual depth and clarity genetic alpha1-antitrypsin deficiency. The progress of our understanding the structure and function of antitrypsin is a remarkable journey from bedside to bench and back. Associated liver disease has a broad clinical spectrum and its pathogenesis is very different from that of pulmonary emphysema and the other associated pulmonary syndromes. Current diagnostic and treatment strategies are meticulously presented to increase awareness, early diagnosis and better patient management.

Hemochromatosis, an iron loading disorder, is a common inherited metabolic disorder. A high index of suspicion leading to early diagnosis of hemochromatosis is crucial since a safe and relatively simple treatment is available. Repeated phlebotomies can restore normal life expectancy if it is introduced before irreversible end-organ damage occurs. The diagnosis of hemochromatosis has therefore to be taken into account in the evaluation of patients with hepatomegaly or elevated liver enzymes. The elegant review by **Antonello Pietrangelo,**

Elena Corradini, and **Francesca Ferrara** from the University of Modena and Reggio Emilia, Italy, summarizes the clinical aspects and the molecular pathogenesis of hemochromatosis. They provide fascinating data on the recently discovered iron hormone that has a central role in the pathogenesis of all forms of hemochromatosis and review the optimal screening and treatment plan for hemochromatosis patients.

Wilson disease is another disease caused by accumulation of metal - copper, in various organs including the liver, cornea and the brain. As in hemochromatosis, a high index of suspicion is crucial, leading to early diagnosis of Wilson disease before serious complications and eventually mortality in untreated cases, occurs. **Peter Ferenci** from the University of Vienna, Austria, a leading figure in the field of Wilson disease, summarizes for us the pathogenesis and clinical presentations and provides insights to the complexity of diagnosis and treatment.

The genetic vulnerability of Ashkenazi Jews to Gaucher disease - the most common lysosomal storage disease, is caused by mutations in the β-glucocerebrosidase gene. **Ari Zimran** and **Deborah Elstein** from the Herew University Hadassah School of Medicine, Jerusalem together with **Stephan vom Dahl** from Cologne, Germany offer a fascinating overview of the molecular biology and clinical results of the accumulation of glucosylceramide in macrophages of the reticuloendothelial system. Since the advent of enzyme replacement therapy for Gaucher disease a decade and a half ago, the quality of life of these patients has dramatically improved. These treatments are now accurately discussed and their future is skillfully outlined.

Joseph Wolfsdorf of Children's Hospital and the Harvard Medical School, Boston and **David Weinstein** currently at the University of Florida, have done an admirable job of discussing glycogen storage diseases (glycogenoses) – the inherited diseases caused by abnormalities of the enzymes that regulate glycogen synthesis or degradation. With much expertise they identify the different mechanisms, epidemiology and treatment for each of the disorders starting with type I glycogen storage disease that is highly amenable to dietary therapy and going on to the ominous (but fortunately rare) type IV glycogen storage disease that can rapidly deteriorate to liver cirrhosis in infancy and responds to liver transplantation alone.

Over twenty different metabolic disorders in children and adults have been treated with liver transplantation, usually in the context of fulminant hepatic failure or advanced disease refractory to medical therapy. **Kris Kowdley** of the University of Washington Medical Center, Seattle, and **Narendra Siddaiah** have undertaken to present an up to date review of this relatively new treatment modality, covering their own experience, as well as the cumulative data from many other groups. Although only about 5% of liver transplantations among adults were performed for metabolic liver diseases, excellent survival rates have been achieved in both pediatric and adult transplantation, making liver transplantation an important treatment modality in life threatening metabolic liver diseases.

Thus 'The Metabolic Aspects of Chronic Liver Disease' presents an opportunity to study an up to date account of all the truly exciting developments and insights in the field, presented by researchers of many nations but a common commitment to excellence and leading contributions in their fields. We are certain that it would prove illuminating and stimulating reading for scientists, clinicians and students alike.

Ami Schattner
Hilla Knobler

Hebrew University Hadassah Medical School
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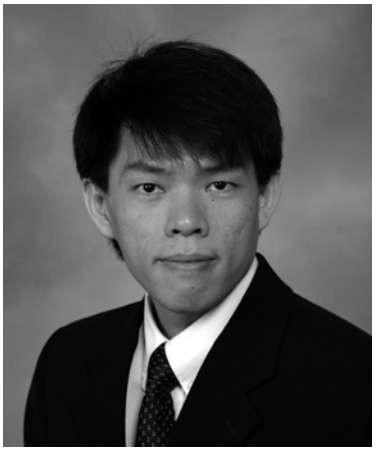
BIOGRAPHICAL SKETCHES OF EDITORS AND CONTRIBUTORS

Professor Bruce R. Bacon, M.D.

Dr. Bruce Bacon graduated the Case Western Reserve University School of Medicine, trained in medicine and gastroenterology and hepatology at the Cleveland Metropolitan General Hospital, joined the faculty at his alma mater, and in 1988, became Chief of the Section of Gastroenterology and Hepatology at Louisiana State University School of Medicine. Moving to Saint Louis University School of Medicine in 1990, Dr. Bacon became the *James F. King, MD Endowed Chair in Gastroenterology*, Professor of Internal Medicine, and Director of the Division of Gastroenterology and Hepatology. Dr. Bacon's research has largely been focused on iron metabolism in the liver. He won the 1989 Marcel Simon Award for best research in hemochromatosis and was elected to the American Society for Clinical Investigation. He has held senior posts at the American Liver Foundation and the NIH, was a member of key editorial boards and the President of the American Association for the Study of Liver Diseases in 2004. Dr. Bacon is co-author of *Essentials of Clinical Hepatology*, co-editor of *Liver Disease: Diagnosis and Management* and of *Comprehensive Clinical Hepatology* and has written more than 295 original articles, reviews, and book chapters.

Metin Basaranoglu, M.D.

Metin Basaronoglu obtained his M.D. degree from Istanbul University School of Medicine and is currently working as a faculty member in the gastroenterology and hepatology division of Selcuk University School of Medicine, Turkey. His primary research interest is the pathogenesis and therapy of non-alcoholic steatohepatitis (NASH) and other areas of interest include viral hepatitis treatment, biliary system disorders and the etiopathogenesis of sarcoidosis. He was twice awarded young investigator travel awards by the American Association for the Study of Liver Diseases to present his research on fatty liver disease.



Phunchai Charatcharoenwitthaya, M.D.

Phunchai Charatcharoenwitthaya, M.D. is a research fellow in the Department of Gastroenterology and Hepatology at Mayo Clinic in Rochester, Minnesota, working with Professor Keith D. Lindor. His research program is focused on management of nonalcoholic steatohepatitis and chronic cholestatic liver disease, including primary biliary cirrhosis and primary sclerosing cholangitis. His major focus has been on clinical trials in these diseases. He is sponsored by an overseas medical fellowship from The Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand.

Elena Corradini, M.D.

Elena Corradini received her degree in Medicine from the University of Modena and Reggio Emilia, Italy, discussing a doctoral thesis on genetics and clinical aspects of the newly discovered ferroportin disease. She then joined the Centre for Hemochromatosis directed by Prof. Antonello Pietrangelo and got involved in basic research studies on the pathogenesis of hemochromatosis. Dr. Corradini has concluded her residency in Internal Medicine in the General Hospital of Modena and is presently a Senior Physician in the out-patient clinic of hemochromatosis patients.

Deborah Elstein, Ph.D.

Deborah Elstein, has been the Coordinator of Clinical Research at the Gaucher Clinic in Shaare Zedek Medical Center (Jerusalem, Israel) since 1993. Prior to moving to Israel in 1979, she did her initial research at Cornell Medical College (NYC) in the field of Pediatric Nephrology. She attended the Hebrew University - Hadassah Medical School (Jerusalem) graduating with a Ph.D. in Medicine in 1983. Her fellowship training from 1984-1987 was in Biochemistry-Biophysics and Molecular Genetics at Columbia-Presbyterian Medical Center (NYC) under the mentorship of Profs. Isidor Edelman and Jurgen Brosius. She is happily married to a dentist and the mother of seven children.

Professor Peter Ferenci, M.D.

Peter Ferenci was born in Budapest, Hungary and graduated from the Medical University of Vienna, Austria in 1972. He trained in internal medicine and Gastroenterology/Hepatology in Vienna and at the National Institutes of Health (Bethesda, MD, USA). His special interests focus on chronic viral hepatitis, genetic liver diseases and hepatic encephalopathy. Since 1990, Dr. Ferenci has been Professor of Medicine at the Medical Faculty of the University of Vienna, Austria. He was also appointed Dr. honoris causae at the University of Cluj Napoca, Romania.

Dr. Ferenci is a member of the American Gastroenterological Association (AGA), the American Association for the Study of Liver Diseases (AASLD), and the European Association for the Study the Liver (EASL). He is the current chairman of the United European Gastroenterology Federation (UEGF). He was President of the Austrian Association of Gastroenterology and Hepatology, 1996–1998, and of the Association des Sociétés Européennes et Méditerranées de Gastroentérologie (ASNEMGE) 2001-2004. He was the program director of the 11th World Congresses of Gastroenterology, Vienna 1998 and the organizer of the 27th Annual Meeting of EASL 1992.

Dr. Ferenci has published over 300 papers and abstracts including authoritative papers on various topics in liver diseases and is the author or editor of one book and of 20 chapters in leading textbooks. He has been an invited lecturer at universities and hospitals throughout the world.

Francesca Ferrara, M.D.

Francesca Ferrara was born in Modena, Italy, and graduated in Medicine in 2000. She completed her residency in Internal Medicine in 2005 and has been working since at the Centre for Hemochromatosis directed by Professor Antonello Pietrangelo, at the University

of Modena and Reggio Emilia. Dr. Ferrara is mainly involved in clinical research and she is presently responsible for the in-patient clinic of primary and secondary hemochromatosis and chronic liver disease.

Yitzchak Halperin, M.D.

Yitzchak Halperin is currently director of the Endocrine unit at Barzili Medical Center in Ashkelon, Israel. Dr. Halperin completed his medical studies at the Hebrew University Hadassah Medical School in Jerusalem, Israel and his residency in internal medicine and endocrinology at the Hadassah Medical Center. Following a fellowship in New York, Dr. Halperin is currently a Senior Lecturer in medicine at the Ben-Gurion University of the Negev Medical School.

Professor Lee M. Kaplan, M.D., Ph.D.

Lee M. Kaplan is Director of the MGH Weight Center and the Obesity Research Center at Massachusetts General Hospital and an Associate Professor of Medicine at Harvard Medical School. Dr. Kaplan graduated from Harvard University and the Albert Einstein College of Medicine. He completed his internship and residency in internal medicine and his

fellowship in gastroenterology at the Massachusetts General Hospital and Harvard Medical School. Dr. Kaplan's clinical expertise is in the areas of gastrointestinal and liver diseases, with a particular focus on fatty liver disease, viral hepatitis and obesity. His current research is focused on the regulation of body weight, the mechanisms of weight loss and improvement in insulin sensitivity after gastric surgery, and the causes and treatment of fatty liver disease.

Professor Hilla Knobler, M.D.

Hilla Knobler was born in Jerusalem. She graduated the Hebrew University and Hadassah Medical School in Jerusalem and did her residency in Internal Medicine in Hadassah University Hospital. During 1991-1993 she did her fellowship in Endocrinology and Metabolism at Mount Sinai Medical Center, New York, where she first became interested in the association between diabetes and chronic hepatitis C infection.

Currently she is the head of the Unit of Metabolic Diseases and Diabetes at Kaplan Medical Center and Clinical Associate Professor of Medicine at the Hebrew University and Hadassah Medical School in Jerusalem. In addition she is a visiting scientist at the Weizmann Institute Rehovot, an active member of the Israel Diabetes Association, the Israeli Diabetes Research Group and the Israeli Society for Research, Prevention and Treatment of Atherosclerosis.

Her current fields of research: glucose metabolism and insulin signal transduction in chronic hepatitis C infection; the role of insulin resistance in NAFLD and in cardiovascular diseases.

Professor Kris V. Kowdley M.D., FACP, FACG, AGAF

Kris V. Kowdley is a Professor of Medicine at the University of Washington School of Medicine in the Division of Gastroenterology and Hepatology. He is the Director and Founder of the Iron Overload Clinic at the University of Washington Medical Center. Dr. Kowdley received his BS in Biology and Anthropology as a member of the Dean's List at Columbia University, and his medical degree from Mount Sinai School of Medicine. He completed his internship and residency at Oregon Health Science University and a Fellowship in Gastroenterology and Hepatology at Tufts University School of Medicine. Dr. Kowdley has presented his research in liver diseases at more than 100 national and international medical centers and scientific symposia. He is the author of over 300 articles, book chapters, reviews and commentaries in this area and has been published in the New England Journal of Medicine, Annals of Internal Medicine, Hepatology, Gastroenterology, Archives of Surgery, Journal of Clinical Gastroenterology and among other professional publications. Dr. Kowdley also serves as a consultant on drug safety for several pharmaceutical and biotechnology companies.

Professor Keith Lindor, M.D.

Keith Lindor, M.D. is a Professor of Medicine in the Department of Gastroenterology and Hepatology at the Mayo Clinic in Rochester, Minnesota and is currently the Dean of the Mayo Medical School. His research program is focused on management of chronic

cholestatic liver diseases, including primary biliary cirrhosis and primary sclerosing cholangitis, and more recently on nonalcoholic steatohepatitis. His major focus has been on clinical trials in these diseases. He has over 200 peer-reviewed publications in this field. His research has been funded by the National Institutes of Health, and he speaks widely on these topics. Prior to assuming the role of Dean of Mayo Medical School, he was Chair of the Division of Gastroenterology and Hepatology.

Professor David Lomas Ph.D. Sc.D FRCP

Professor David Lomas PhD ScD FRCP FMed Sci qualified from the University of Nottingham in 1985 and then worked in Nottingham and Birmingham as a junior hospital doctor in General and Respiratory Medicine. He moved to Cambridge as an MRC Training Fellow to undertake his PhD and then secured a second fellowship as an MRC Clinician Scientist. In 1994 he was appointed as University Lecturer at the University of Cambridge and in 1998 was appointed to the Professorship of Respiratory Biology. He has been an Honorary Consultant Respiratory Physician at Addenbrooke's and Papworth Hospitals in Cambridge since 1994 and has a particular interest in α_1-antitrypsin deficiency, the serpinopathies and the genetic basis of emphysema.

Meera Mallya B.Sc. (Hons) Ph.D.

Dr Meera Mallya qualified in Biochemistry from Imperial College (University of London) in 1999 and then obtained her Ph.D. in Cambridge in 2003 studying Molecular

Biology at the Wellcome Trust Genome Campus in Hinxton. She is currently employed in the laboratory of Professor David Lomas as a Post Doctoral Research Fellow in the University of Cambridge, where she is working on the structural biology of 1-antitrypsin, in particular strategies to prevent polymerisation of Z α_1-antitrypsin and has obtained a European α_1-Antitrypsin Laurell's Training Award (ALTA) fellowship.

Stephen Malnick, **M.A. (Oxon) M.sc. MBBS (Lond)**

Stephen Malnick is currently Director of the Department of Internal Medicine C at Kaplan Medical Center in Rehovot, Israel and a Senior Lecturer in medicine at Hadassah Medical School, Hebrew University in Jerusalem. Dr Malnick graduated from Oriel College, Oxford and completed his medical studies at Middlesex Hospital, London, England. He completed his internal medicine residency and gastroenterology fellowship at Kaplan Medical Center. Dr Malnick's clinical specialty is in the area of treatment of viral hepatitis and non-alcoholic fatty liver disease (NAFLD). His research interests focus on the clinical aspects of NAFLD and the effects of obesity on the heart.

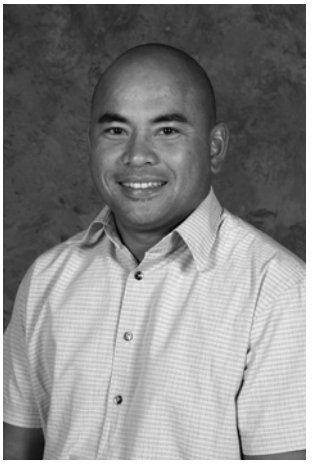
Professor Brent A. Neuschwander-Tetri, M.D., FACP

Brent A. Neuschwander-Tetri completed his undergraduate studies at the University of Oregon and received his M.D. degree from Yale University. He completed his internship and residency in internal medicine at the University of Wisconsin Madison and went on to a

fellowship in gastroenterology and liver diseases at University of California San Francisco. In 1991 he joined the faculty at Saint Louis University in the Division of Gastroenterology and Hepatology where he directs the teaching of the basic science of gastroenterology and hepatology to medical students, conducts clinical research in nonalcoholic steatohepatitis and basic research in pancreatic fibrogenesis.

Professor John K. Olynyk, M.D.

Professor John Olynyk is a Gastroenterologist and Hepatologist based in the School of Medicine and Pharmacology at Fremantle Hospital. He has been in his current position since April 1994. He has established major research programs in the broad areas of colorectal cancer screening, pathogenesis of hereditary haemochromatosis and the role of hepatic stem cells in the pathogenesis of liver cancer. His research is funded by NH&MRC and the Cancer Foundation of Western Australia.



John Ombiga, M.D.

Dr. John Ombiga is a Gastroenterology Research Fellow based at Fremantle Hospital since January 2005 where he has been involved with research in inflammatory bowel disease,

and the publication of an important review on screening for the HFE gene in hereditary haemochromatosis and iron overload.

Russell Phillips BSc (Hons) MB; BS (Hons), MRCP (Lond)

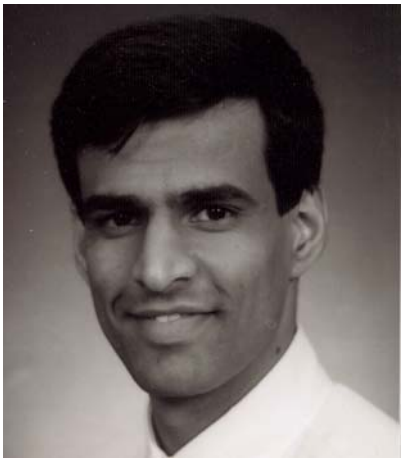
Dr. Russell Phillips qualified from the Royal Free Hospital Medical School (University of London) in 1997 and then worked in London and Cambridge as a junior hospital doctor before becoming a Specialist Registrar in Respiratory and General Internal Medicine in Cambridge and the East Anglia region in 2001. As part of his training he is currently undertaking a Ph..D. in the laboratory of Professor David Lomas in the University of Cambridge where he is working with Dr. Meera Mallya on the structural biology of α1-antitrypsin deficiency having been awarded a Wellcome Trust Clinical Research Fellowship.

Professor Antonello Pietrangello, M.D., Ph.D.

Antonello Pietrangello, M.D., Ph.D. was born in 1956. He graduated cum laude the University of Modena where he later trained in Gastroenterology and completed his PhD (cum laude). He spent several years at the Liver Research Center of the Albert Einstein College of Medicine, New York and came back to the University of Modena and Reggio

Emilia where he is Professor of Medicine since 2001 and Chief of the Center for Hemochromatosis and Hereditary Liver Diseases at the University Hospital of Modena.

In addition to his being the senior author on numerous publications, Dr. Pietrangello received the NATO Advanced Fellowship award (1989), the Fogarty International Fellowship Award (1990), the International Young Investigator Award of the European Association for the Study of Liver Diseases (EASLD) (1993), the Marcel Simon Award for excellence in research in disorders of iron metabolism (1995) and the EASLD International Investigator award (1996). He was also the chairman of the World Iron Congress in 1999, president of the International Bioiron Society (IBIS) and member of the Scientific Committee of the EASLD. His main fields of interest are iron metabolism and hemochromatosis; molecular and cell biology of oxidant stress, inflammation and fibrosis in liver diseases and hepatic gene expression and gene therapy.



Narendra Siddaiah, M.D.

Narendra Siddaiah, M.D. is a Research Fellow in Hepatology at the University of Washington School of Medicine, Division of Gastroenterology and Hepatology. He completed Internship and Residency in Internal Medicine at St. Francis Hospital in Evanston, Illinois and has practiced and taught Internal Medicine. Dr. Siddaiah received his M.B.B.S degree from Bangalore University in India and pursued graduate studies in Immunology at the University of Saskatchewan, Canada. He will begin his clinical fellowship in Gastroenterolgy and Hepatology at the University of Mississippi Medical Center.

Professor Ami Schattner, M.D.

Ami Schattner was born in Haifa, Israel to parents who emigrated from Europe days before the war and the Holocaust. He graduated from the Hebrew University and Hadassah Medical School in Jerusalem (1974) where he is now an Associate Professor of Medicine and a distinguished teacher. After his residency in Internal Medicine he gained research experience at the Department of Virology of the Weizmann Institute of Science in Rehovot, and was a Fulbright Fellow at the Albert Einstein School of Medicine in New York and later at Tufts University Medical School, Boston. He is currently Chief (since 1991) of a Department of Medicine at the Kaplan Medical Center, a Hadassah Medical School teaching hospital in Rehovot. Dr. Schattner has spent Sabbaticals as a Visiting Professor at Stanford (1996), Harvard (2001) and Cambridge (2004) Universities and has initiated many research projects and authored numerous publications and book chapters. His main research interests are cytokines in autoimmunity, autoimmune diseases, hepatitis C-induced cytokines and their effects on the liver and on insulin resistance, patient-physician relationship and the quality of care.

Debbie Trinder, Ph.D.

Dr Debbie Trinder is a Senior Research Fellow in the University of Western Australia, School of Medicine and Pharmacology at Fremantle Hospital, Perth, Western Australia. Her main research interests are liver iron metabolism and hereditary haemochromatosis.

Professor Stephan vom Dahl, **M.D.**

Stephan vom Dahl graduated from Medical School of University of Dusseldorf in Germany in 1989. His medical education included the universities of Freiburg, Duesseldorf and the University of Pennsylvania, Philadelphia. He is an internist and gastroenterologist who became Associate Professor of internal medicine and hepatology at the University of Duesseldorf in 2001. Since 2005 he is Chief of the Department of Internal Medicine at St. Franziskus-Hospital, Cologne. His basic research interests are the regulation of liver metabolism, and his clinical fields include metabolic liver diseases and Gaucher disease. He has published numerous articles on regulation of liver metabolism and clinical issues of Gaucher disease.

Professor David A. Weinstein, M.D., M.M.Sc.

David A. Weinstein graduated from Trinity College (CT) and Harvard Medical School, and then completed a residency, chief residency, and fellowship in pediatric endocrinology at Children's Hospital, Boston. He subsequently obtained a Masters in clinical investigation from Harvard and MIT, and became Director of the Glycogen Storage Disease Program at Children's Hospital Boston. In 2005, Dr. Weinstein moved to the University of Florida where

he directs the Glycogen Storage Disease Program and is an Associate Professor of Pediatrics. Dr. Weinstein follows one of the largest cohorts of GSD patients in the world, and he directs a research team investigating novel therapies for the glycogen storage diseases. He is a former Jan Albrecht Award winner from the American Association for the Study of Liver Diseases, and he is on the Board of Directors for the Association for Glycogen Storage Disease.

Professor Joseph I. Wolfsdorf, M.B., B.Ch.

Dr. Joseph I. Wolfsdorf received his medical education from the University of Witwatersrand in Johannesburg, South Africa, from which he graduated with an M.B., B.Ch. in 1969. He was a registrar in pediatrics at Baragwanath Hospital and the Transvaal Memorial Hospital for Children from 1972-1975. After obtaining a Diploma in Child Health in 1973 and the Fellowship of the College of Physicians of South Africa (with Pediatrics) in 1974, he emigrated to the United States of America in 1975. From 1975-1976, he was a Fellow in Pediatric Endocrinology at the University of Chicago, and from 1976-1978 a Clinical and Research Fellow in Pediatric Endocrinology and Metabolism, at Tufts-New England Medical Center in Boston, where he developed an interest in glycogen storage diseases while working with Dr. Boris Senior. In 1982, he began to work on glycogen storage disease with Dr. John F. Crigler, Jr., at Children's Hospital Boston. Dr. Wolfsdorf is Associate Chief of the Division of Endocrinology, Director of the Diabetes Program, and an Associate Professor of Pediatrics at Harvard Medical School.

Professor Ari Zimran, M.D.

Ari Zimran graduated from the Hebrew University, Hadassah Medical School, Jerusalem, Israel in 1975. He served several years as a medical officer in the Israeli army, prior to completion of his residency in Internal Medicine at Shaare Zedek Medical Center in Jerusalem in 1986. During 3 years of research fellowship at the Scripps Research Institute in La-Jolla, under the mentorship of Prof. Ernest Beutler, he became interested in both molecular and clinical aspects of Gaucher disease. Upon return to Israel he founded a referral center for patients with Gaucher disease, where over 600 patients with Gaucher disease are being followed. Dr. Zimran participated in several clinical trials that led to market approval of new treatments for patients with Gaucher disease, both multi-center and single center studies. He published over 150 papers and edited two books - one on Gaucher disease and the other on Lysosomal Storage Disorders.

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Chapter I

PATHOPHYSIOLOGY OF NASH

Metin Basaranoglu[1] and Brent A. Neuschwander-Tetri[2,*]

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[2]Saint Louis University Liver Center and Division of Gastroenterology and Hepatology,
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ABSTRACT

Rapid advances on molecular studies, manipulation of the mouse genome, the development of a number of animal models, and using these in studies of nonalcoholic fatty liver disease (NAFLD) have provided important insights into the pathogenesis of this relatively common disorder. One of the most crucial advances was to recognize the links among obesity, insulin resistance, inflammation and NAFLD. A growing body of literature has shown that insulin resistance and its liver-related consequence, NAFLD, could be the result of generalized inflammation. Genetic and behavioral factors contribute to increased visceral adipose tissue where increased oxidative stress and lipid peroxidation may contribute to dysregulated production of adipocytokines, fatty acids, and bioactive lipids. This chain of these events may contribute to local and peripheral insulin resistance, a central underlying pathophysiological process that may both cause and result from increased peripheral lipolysis and elevated free fatty acid concentrations in the circulation. Abnormally elevated free fatty acids taken up by organs other than adipose tissue, such as liver and skeletal muscle, contributes to steatosis of these organs (ectopic lipogenesis). Increased muscle and hepatocellular lipid content provides substrates for oxidative stress and lipid peroxidation, and also promotes insulin resistance in both liver and muscle by disturbing their downstream insulin signaling cascades. Insulin resistance further increases peripheral lipolysis in adipose tissue, further elevates circulating free fatty acids, inhibits hepatic fatty acid β-oxidation and increases de novo

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synthesis of both fatty acids and triglycerides in the liver. Excessively produced triglycerides in the liver are either stored as fat droplets or secreted into the plasma as very-low-density lipoproteins. If this complex mechanism of hepatic fat synthesis and secretion capacity is overwhelmed, excessive triglycerides accumulate within the hepatocytes and manifests as NAFLD.

A fatty liver is sensitive to hepatocellular injury and sustained injury can manifest as nonalcoholic steatohepatitis (NASH), NASH-associated cirrhosis, and NASH-associated hepatocellular carcinoma. Specific depletion of hepatic natural killer T cells with consequent proinflammatory cytokine polarization of liver cytokine production might be one reason for this increased hepatic sensitivity against various stimuli. Only a minority of patients with NAFLD have the necroinflammatory changes of NASH. The development of NASH in patients with NAFLD may be the consequence of secondary abnormalities such as injured and dysfunctional mitochondria, generation of reactive oxygen species with down-regulation or consumption of antioxidants causing oxidative stress and lipid peroxidation, increased activity of cytochrome P450 2E1, disturbed production of adipocytokines, and the effects of gut-derived cytotoxic products. The dynamic interplay of these processes in the pathogenesis of NAFLD remains incompletely understood and is an area of active research.

Keywords: nonalcoholic steatohepatitis, insulin resistance, fatty acids, adipocytokines, CYP2E1, oxidative stress, mitochondrial dysfunction.

ABBREVIATIONS

AdipoR, adiponectin receptor; αSMA; α-smooth muscle actin; AOX, acyl-CoA oxidase; apoB 100, apolipoprotein B100; APS, adaptor protein with a PH (pleckstrin homology) and SH2 (Src homology 2) domain; BMI, body mass index; ChREBP, carbohydrate response element binding protein; CIS, cytokine-inducible src homology 2 domain-containing protein; CPT, carnitine palmitoyltransferase; CRP, C-reactive protein; CTGF, connective tissue growth factor; CYP, cytochrome P450; DNL, de novo lipogenesis; ECM, extracellular matrix components; GLUT, glucose transporter; HCC, hepatocellular carcinoma; HSC, hepatic stellate cells; HSP, heat shock protein; JNK, c-Jun N-terminal kinase; *HFE*, hemochromatosis gene; IDL, intermediate density lipoproteins; IKK-β, inhibitor κB kinase β; IL, interleukin; iNOS, inducible nitric oxide synthase; IRS, insulin receptor substrate; LPS, lipopolysaccharide; LXR-α, liver X receptor- α; MAPK, mitogen-activated protein kinase; MCD, methionine-choline deficient; MMC, megamitochondria with true crystalline inclusions; MRC, mitochondrial respiratory chain; MTP, mitochondrial trifunctional protein; MTTP, microsomal triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NEFA, non-esterified fatty acids; NF-κB, nuclear factor kappa B; NKT cells, natural killer T cells; NOS2, nitric oxide synthase-2; PERPP, postendoplasmic reticulum presecretory proteolysis; PI3-K, phosphatidyl inositol 3-kinase; PKB, protein kinase B; PKCδ, protein kinase C delta; PKCε, protein kinase C epsilon; PKCλ, protein kinase C lamda; PKCθ, protein kinase C theta; PKCξ, protein kinase C XI; PPAR, peroxisome proliferator-activated receptor; PUFAs, polyunsaturated fatty acids; r-metHuLeptin; recombinant methionyl human leptin; ROS, reactive oxygen species; Ser,

serine; Shc, Src homology collagen; SOCS, suppressors of cytokine signaling; SREBP-1c, sterol regulatory element-binding protein-1c; STAT-3, signal transduction and activator of transcription-3; TBARSs, thiobarbituric acid-reactive substances; TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor-β; UCP, uncoupling protein; VLDL, very-low-density lipoprotein; WAT, white adipose tissue.

PATHOPHYSIOLOGY OF EXCESSIVE FAT ACCUMULATION IN THE LIVER IN THE ABSENCE OF ALCOHOL ABUSE: NAFLD

Excessive accumulation of triglycerides in hepatocytes in the absence of significant alcohol consumption, defined as > 5% fat by weight, [1,2] occurs in about 20-30% of adults [3-8]. Excessive fat in the liver, called nonalcoholic fatty liver disease or NAFLD, predisposes to the development of nonalcoholic steatohepatitis (NASH) [1,2]. NASH constitutes the subset of NAFLD that is most worrisome because it is a significant risk factor for developing cirrhosis and its complications, including hepatocellular carcinoma (HCC) (Table 1) [9-17]. Because the accumulation of excess fat in the liver is a prerequisite for the development of NASH, understanding the underlying causes of NAFLD forms the basis for rational preventive and treatment strategies of this major form of chronic liver disease. Insulin resistance and hyperinsulinemia are the most common underlying abnormalities in people with NAFLD.

Table 1. Terminology of NAFLD.

NAFLD: an inclusive term for liver disease characterized by predominantly macrovesicular steatosis in which hepatocytes contain vacuoles of triglyceride
Benign or simple steatosis: the generally non-progressive form of NAFLD
NASH: the progressive form of NAFLD that also includes significant necroinflammatory changes and variable degrees of fibrosis
NASH-associated subacute liver failure
NASH-associated cirrhosis: may lose the histological features of NASH
NASH-associated HCC

Obesity, Insulin Resistance and Hyperinsulinemia as Risk Factors for NAFLD

Overwhelming evidence now indicates that identifying NAFLD in a patient is a sensitive surrogate marker for the presence of underlying insulin resistance in most patients [18-27]. Ideally, a balance exists between energy demand and intake in the human body. Overnutrition (obesity) and starvation are the two major abnormalities of this well preserved equilibrium. Obesity, and its consequences such as insulin resistance and the metabolic syndrome (Table 2), is a growing threat to the health of people in developed nations [27-30]. While insulin

receptor defects cause severe insulin resistance, most patients with insulin resistance have impaired post-receptor intracellular insulin signaling. Moreover, there is a cross-talk among insulin sensitive tissues. For example, a single genetic defect in one insulin target tissue could result in insulin resistance in other tissues [29]. Understanding the causes and consequences of these defects is the focus of intense investigation to better understand the pathophysiology of type 2 diabetes mellitus, a common consequence of decades of insulin resistance.

Table 2. The metabolic syndrome is present when three or more of five criteria are met [422].

Abdominal obesity: waist circumference > 40 inches (men) or > 35 inches (women)
Elevated fasting glucose: ≥ 100 or treatment of elevated glucose
Elevated blood pressure: systolic ≥ 130 mm Hg or diastolic ≥ 85 mm Hg or treatment of hypertension
Elevated triglycerides: ≥ 150 mg/dL or treatment of elevated triglycerides
Low HDL-cholesterol: < 40 mg/dL (men) or < 50 mg/dL (women) or treatment

Insulin binds α-subunits of its receptor which is a cell surface receptor on the major insulin sensitive cells such as skeletal muscle, adipocytes, and hepatocytes leading to autophosphorylation of the cytoplasmic domains (β-subunits) of the receptor [29-33]. The insulin receptor has intrinsic tyrosine kinase activity activated by insulin binding and the autophosphorylated receptor activates its substrates that included insulin receptor substrate (IRS) -1, IRS-2, Shc (Src homology collagen), and APS (adaptor protein with a PH [pleckstrin homology] and SH2 [Src homology 2] domain) by tyrosine phosphorylation. These phosphorylated docking proteins bind and activate several downstream components of the insulin signaling pathways. For example, tyrosine phosphorylated Shc, with Grb2-SOS, activates mitogen-activated protein kinase (MAPK) cascade. MAPK regulates gene expression and is involved in cellular growth. Activated IRS-1 associates with phosphatidyl inositol 3-kinase (PI3-K), which then activates Akt. In both skeletal muscle and adipose tissue, these insulin-mediated phosphorylation-dephosphorylation signaling cascades induce the translocation of glucose transporters (GLUT), predominantly GLUT4 -containing vesicles, from intracellular storage sites to the plasma membrane, increasing glucose uptake to prevent abnormal glucose and insulin elevations in the plasma (insulin-stimulated glucose transport). These events and insulin-dependent inhibition of hepatic glucose output maintain glucose homeostasis. Insulin also affects glucose homeostasis indirectly by its regulatory effect on lipid metabolism. Any interference in this insulin signaling pathway causes glucotoxicity, insulin resistance and, when islet beta cells are capable of responding, compensatory hyperinsulinemia.

Hepatic expression of insulin receptor protein in humans and the levels of both IRS-1 and IRS-2 in animals were decreased in chronic hyperinsulinemic states [34-36]. Interestingly, near total to total ablation of insulin receptor protein expression in the liver (up to 95%) did not alter the hepatic glucose production in mice [36] while liver-specific insulin receptor deficient mice showed both insulin resistance and glucose intolerance [37]. It was also demonstrated in mice that hepatic IRS-1 and IRS-2 play complementary roles in the

regulation of hepatic metabolism. IRS-1 was more closely linked to glucose homeostasis with the regulation of glucokinase expression while IRS-2 was more closely linked to the lipogenesis with the regulation of lipogenic enzymes SREBP-1c (sterol regulatory element-binding protein-1c) and fatty acid synthase [35].

Additional physiological roles of insulin include regulating the metabolism of macronutrients and stimulating cellular growth (Figure 1). Insulin activates synthesis and inhibits catabolism of lipids while shutting off the synthesis of glucose in the liver. Adipose tissue is one of the major insulin sensitive organs in human body and the process of differentiation of preadipocytes to adipocytes, induced by insulin, is called as adipogenesis [30,31,38-42]. Within the adipose tissue, insulin stimulates triglyceride synthesis (lipogenesis) and inhibits lipolysis by upregulating lipoprotein lipase activity which is the most sensitive pathway in insulin action, facilitating free fatty acid uptake and glucose transport, inhibiting hormone sensitive lipase, and increasing gene expression of lipogenic enzymes. Insulin also induces the degradation of apolipoprotein B100 (apoB 100), a key component of very-low-density lipoprotein (VLDL), in the liver [38].

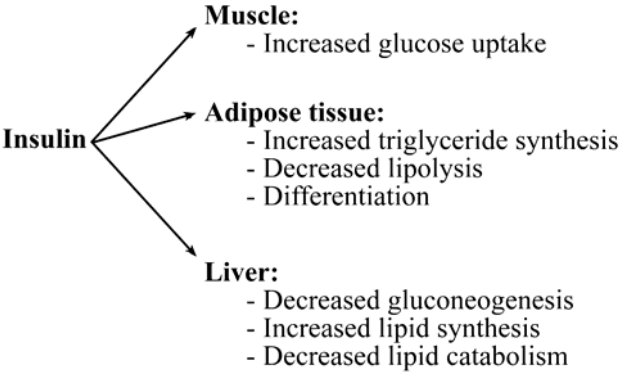

Figure 1. The major functions of insulin. Muscle, adipose tissue and the liver are the major targets of circulating insulin. Elevated insulin levels in the fed state effect a major change in whole body metabolic processes from gluconeogenesis and breakdown of fat to glucose uptake and disposal by formation of glycogen and fat while shutting off the catabolism of fat. In muscle, insulin promote glucose uptake by increasing the membrane expression of the glucose transporter GLUT4. In adipose tissue, triglyceride synthesis is increased as lipolysis and formation of free fatty acids is shut off. In the liver, gluconeogenesis and mitochondrial β-oxidation are shut off while synthesis of fatty acids and triglyceride are upregulated. These processes are impaired in the insulin resistant state such that muscle inadequately removes glucose from the circulation, adipose tissue continues to release free fatty acids even in fed state and the liver must handle this excess of fatty acids. GLUT4: glucose transporter 4.

Insulin resistance can be defined as the failure of insulin sensitive cells to respond to insulin normally. It is characterized by elevated plasma glucose and, before attrition of pancreatic β-cells develops, elevated insulin levels. Chronic hyperinsulinemia is a major contributor to glucose and lipid metabolism abnormalities. Insulin resistance diminishes the inhibitory effect of insulin on hepatic glucose output and causes impaired insulin mediated glucose uptake in both skeletal muscle and adipocytes [30,43,44]. Insulin resistance also inappropriately activates peripheral lipolysis and stimulates free fatty acid mobilization from adipocytes in the fed state. Increased circulating free fatty acids contribute to fat

accumulation in the liver and muscle, further causing these tissues to be insulin resistant via disturbing their downstream insulin signaling cascades.

Cellular Mechanisms of Insulin Resistance

The most common mechanism of insulin resistance is disturbed post-receptor insulin signaling (Figure 2) [29-32,45,46]. Whereas most insulin signaling is propagated by tyrosine phosphorylation, serine (Ser) phosphorylation is often inhibitory. Ser phosphorylation of IRS-1 decreases both insulin stimulated tyrosine phosphorylation of IRS-1 (phosphorylated Ser residues of IRS-1 become poor substrates for insulin receptor) and PI3-K activation. This diminishes the downstream insulin signaling and insulin sensitivity of insulin target tissues. IRS-1 has several Ser residues such as Ser 307, Ser 612, and Ser 632 which can be phosphorylated. Prolonged insulin stimulation also causes phosphorylation of Ser residues of IRS-1 under physiological conditions [32]. Insulin and tumor necrosis factor-alpha (TNF-α) could phosphorylate the same Ser residues of IRS-1.

TNF-α and plasma free fatty acids have been shown to be major stimuli of Ser 307 phosphorylation of IRS-1 [29-32,45-49]. Inhibition of IRS-1 due to the phosphorylation of its Ser 307 residues also requires the activation of both c-Jun N-terminal kinase (JNK) and inhibitor κB kinase β (IKK-β). Both TNF-α and free fatty acids induce JNK and IKK-β activation.

TNF-α stimulates phosphorylation of Ser residues of both IRS-1 and IRS-2 in hepatocytes [46,50,51] and Ser residues of IRS-1 in muscles [47]. It was recently reported that monocyte-derived macrophages increasingly accumulated within adipose tissue of obese patients. In addition to the dysregulated production of adipocytokines by adipocytes, adipose tissue macrophages also produce proinflammatory cytokines such as TNF-α and interleukin-6 (IL-6), and C-reactive protein (CRP). Both adipose tissue and its macrophages contribute to the TNF-α burden. TNF-α functions in both an autocrine and paracrine manner. Indeed, its circulating concentrations are very low, commonly undetectable even in obese mice or humans. Thus, TNF-α may exert primarily local effects rather than distant effects [52].

Elevated free fatty acids in the circulation are also major contributors to insulin resistance in both humans and mice by stimulating Ser 307 phosphorylation of IRS-1. Adipose tissue triglycerides are the main source of circulating free fatty acids in obese. One mechanism of elevated free fatty acid-induced insulin resistance in muscle is the impaired activation of PKCλ (protein kinase C lamda) and PKCξ (protein kinase C XI) [53]. PKCδ (protein kinase C delta) and β2 might also play roles in human muscle insulin resistance. Additionally, PKCδ is reported as a possible mediator of fatty acid-induced hepatic insulin resistance [54]. In contrast, PKCε (protein kinase C epsilon), not PKCδ, is reported as a possible mediator for fatty acid-induced hepatic insulin resistance in rats (see below) [55]. Diacylglycerol, a metabolic product of long chain acyl CoAs, activates PKCθ (protein kinase C theta) which phosphorylates Ser 307 residues of IRS-1 and subsequently causes skeletal muscle insulin resistance in rodents [56]. PKCθ could also activate IKK-β which phosphorylates Ser 307 residues of IRS-1. Additionally, increased acyl CoAs or ceramide which is a derivative of acyl CoAs, promote insulin resistance by diminishing Akt1 activation [57]. Increased ceramide activates a phosphatase (protein phosphatase 2A) that reverses tyrosine phosphorylation of Akt/protein kinase B (PKB). Inactivated PKB inhibits insulin

downstream signaling cascade and leading to insulin resistance in muscles [32]. It was shown in the liver of rats fed high-fat diet that activation of PKCε and JNK-1 caused the inactivation of IRS-1 and IRS-2, and eventually insulin resistance [55]. Human studies in insulin resistant patients with obesity or diabetes also pointed out a mitochondrial oxidative phosphorylation defect. Moreover, this defect was found associated with the accumulation of triglycerides in muscle [58]. Several oxidative stress mediators might also induce insulin resistance by affecting insulin downstream signaling.

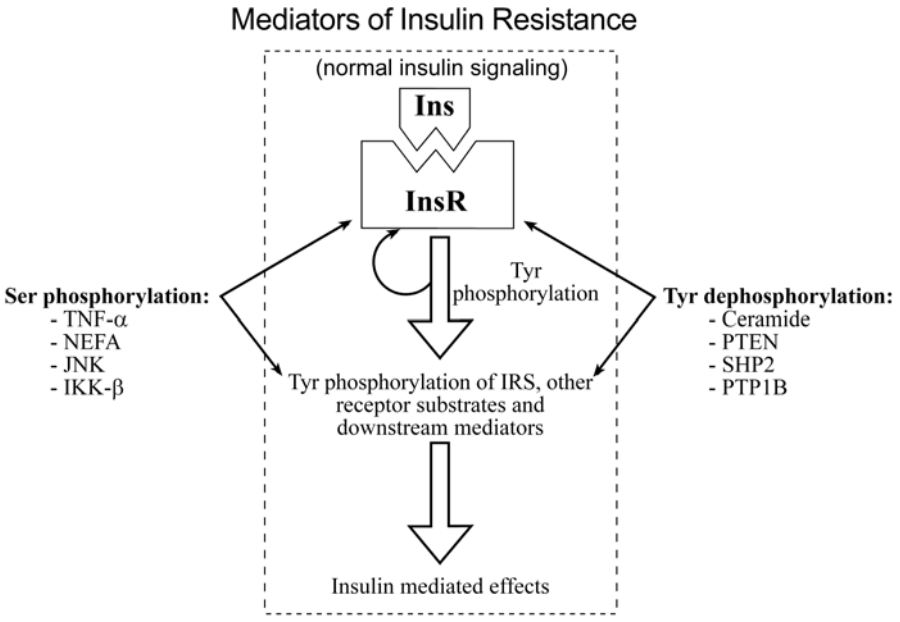

Figure 2. Major mechanisms of insulin resistance. Insulin resistance is most commonly caused by post-receptor signaling defects. The insulin receptor is a tyrosine kinase that autophosphorylates itself and also phosphorylates tyrosine residues on multiple other proteins that participate in signal transduction of the insulin binding such as the insulin receptor substrate (IRS) molecules, Shc, and APS and further downstream mediators such as PI3-K and AkT. Such tyrosine phosphorylation is required for transmitting the signal of insulin binding through the cascade of post-receptor molecules. The phosphotyrosines are dephosphorylated by a number of phosphatases, a process that is normally needed to shut off insulin signaling but can be inappropriately activated to cause insulin resistance. The receptor and the other post-receptor molecules can also be phosphorylated on serine residues, and serine phosphorylation generally impairs the functions of these proteins in transmitting the insulin signal and is a major cause of insulin resistance. Ins: insulin; InsR: insulin receptor; IRSs: insulin receptor substrates; Tyr: tyrosine; Ser: serine; TNF-α: tumor necrosis factor alpha; NEFA: non-esterified free fatty acids; JNK: c-Jun N-terminal kinase; IKK-β: inhibitor IκB kinase; PTEN: phosphatase and tensin homolog deleted on chromosome ten; SHP2: Src homology 2 containing protein tyrosine phosphatase 2; PTP1B: protein tyrosine phosphatase 1B; PI3-K: phosphatidyl inositol 3-kinase; APS: adaptor protein with a PH (pleckstrin homology) and SH2 (Src homology 2) domain; Shc: Src homology collagen.

Phosphatases such as PTEN, SHP 2, and PTP 1B are now recognized to be major mediators involved in insulin resistance. They dephosphorylate activated PI3-K, IRS, and the insulin receptor, respectively to induce insulin resistance. Another possible mechanism for

insulin resistance is defective glucose transport such as down-regulation of GLUT4 (see above) [59].

JNK is one of the stress related kinases and plays an important role in the development of insulin resistance [46,60,61]. The three members of the JNK group of serine/threonine kinases, namely JNK-1, -2, and -3 are activated by proinflammatory cytokines such as TNF-α as well as free fatty acids and endoplasmic reticulum stress due to metabolic overload which is an intracellular abnormality found in obesity. Activated JNK induces Ser 307 phosphorylation of IRS-1, disturbs insulin downstream signaling, and subsequently causes insulin resistance. JNK activity has been found to be elevated in liver, muscle, and adipose tissue of obese experimental models [46]. Additionally, the loss of JNK-1 activity such as in JNK-1 knockout mice has been shown to prevent the development of insulin resistance in leptin deficient *ob/ob* mice or mice with high-fat induced dietary obesity.

Proinflammatory Signaling and Insulin Resistance

PKCθ and IKK-β are two proinflammatory kinases involved in insulin downstream signaling [60,61]. They are activated by lipid metabolites such as high plasma free fatty acid concentrations and there is a positive relationship between the activation of PKCθ and the concentration of intermediate fatty acid products. PKCθ activates both IKK-β and JNK, leading to increased Ser 307 phosphorylation of IRS-1 and insulin resistance. IKK-β is a mediator of insulin resistance and one of the other stress related kinases [45,62-64]. Activation or overexpression of IKK-β diminishes insulin signaling and causes insulin resistance whereas inhibition of IKK-β improves insulin sensitivity. Inhibition of IKK-β activity prevented insulin resistance due to TNF-α in cultured cells. Moreover, high-dose salicylates inhibited IKK-β activation and subsequently reversed insulin resistance in *ob/ob* mice and obese mice by a high-fat diet [45,63]. Mice heterozygous for IKK-β deletion are also partially protected against insulin resistance caused by intravenous lipid infusions, high fat diet, or genetic obesity. Evidence that this process is relevant to human disease was provided by the observation of improved insulin signaling in diabetic patients in whom high-dose aspirin inhibited IKK-β activation [65]. IKK-β phosphorylates the inhibitor of nuclear factor kappa B (NF-κB) leading to the activation of NF-κB by the translocation of NF-κB to the nucleus. NF-κB is an inducible transcription factor and promotes specific gene expression in the nucleus. For example, NF-κB regulates the production of multiple inflammatory mediators such as TNF-α and IL-6 [66]. TNF- α and reactive oxygen species (ROS) could also activate NF-κB. In contrast, antioxidants inhibit this activation. NF-κB has both apoptotic and anti-apoptotic effects. The finding that NF-κB deficient mice were protected from high-fat diet induced insulin resistance suggests that NF-κB directly participates in processes that impair insulin signaling. High-dose salicylates also inhibit NF-κB and subsequently improve insulin sensitivity. Moreover, Cai and colleagues demonstrated that lipid accumulation in the livers of obese mice due to high-fat diet led to subacute hepatic inflammation through activated NF-κB and activation of its targets, such as up-regulation of proinflammatory cytokines [66]. These subsequently promoted hepatic and systemic insulin resistance. Additionally, ROS-induced early NF-κB activation might increase the production of inflammatory mediators and cause steatohepatitis in a methionine-choline deficient (MCD) diet fed animal model [67]. The same study group also showed that these results were

reversed by curcumin which inhibits NF-κB activity. Curcumin also has the ability to induce antioxidant enzymes and scavenge ROS.

SOCS (suppressors of cytokine signaling) and iNOS (inducible nitric oxide synthase) are two inflammatory mediators recently recognized to play a role in insulin signaling [68-70]. Induction of SOCS proteins (SOCS 1-7 and cytokine-inducible src homology 2 domain-containing protein [CIS]) by proinflammatory cytokines might contribute to the cytokine mediated insulin resistance in obese subjects [68-73]. In fact, the isoforms of SOCS are the members of a negative feedback loop of cytokine signaling, regulated by both phosphorylation and transcription events. SOCS-1 and particularly SOCS-3 are involved in the inhibition of insulin signaling either by interfering with IRS-1 and IRS-2 tyrosine phosphorylation or by the degradation of their substrates. SOCS-3 might also regulate central leptin action and play a role in the leptin resistance of obese human subjects [74]. SOCS might be a link between leptin and insulin resistance because insulin levels are increased in leptin resistant conditions due to the diminished insulin suppression effect of leptin because of insufficient leptin levels. Moreover, SOCS proteins might involve insulin/insulin like growth factor-1 signaling. SOCS-1 knockout mice showed low glucose concentrations and increased insulin sensitivity. In animal studies, inactivation of SOCS-3 or SOCS-1 or both in the livers of *db/db* mice partially improved insulin sensitivity and decreased hyperinsulinemia whereas overexpression of SOCS-1 and SOCS-3 in obese animals caused insulin resistance and also increased activation of SREBP-1c [70]. SREBP-1c is one of the key mediators of lipid synthesis from glucose and other precursors (de novo lipogenesis) in the liver [75]. Indeed, SOCS proteins markedly induce de novo fatty acid synthesis in the liver by both the up-regulation of SREBP-1c and persistent insulin resistance with hyperinsulinemia which stimulates SREBP-1c-mediated gene expression. These eventually cause NAFLD. Liver is the insulin clearance organ. Thus, decreased insulin clearance in patients with NAFLD further elevates insulin levels in the circulation and de novo lipogenesis rate in the liver. SOCS-1 and SOCS-3 may exert these effects by inhibiting signal transduction and activator of transcription proteins (STAT), particularly STAT-3, via binding JAK tyrosine kinase because this binding diminishes phosphorylation ability of JAK kinase to STAT-3. STAT-3 inhibits the activation of SREBP-1c. Specific STAT-3 knockout mice showed markedly increased expression of SREBP-1c and subsequently increased fat content in the liver. Conversely, inhibition of SOCS proteins, particularly SOCS-3 improved both insulin sensitivity and the activation of SREBP-1c which eventually reduced liver steatosis and hypertriglyceridemia in *db/db* mice. These results had been achieved by the improvement of STAT-3 phosphorylation and subsequently normalization of the upregulated expression of SREBP-1c [70].

Nitric oxide synthase-2 (NOS2) or iNOS production are also induced by proinflammatory cytokines [61,76,77]. High-fat diet in rats causes up-regulation of iNOS mRNA expression and increases iNOS protein activity [78]. Increased production of NOS2 might reduce insulin action in both muscle and pancreas and decreased iNOS activity protects muscles from the high-fat diet induced insulin resistance. It was also shown that leptin deficient *ob/ob* mice without iNOS were more insulin sensitive than *ob* wild-type. Thus, the production of nitric oxide may be one link between inflammation and insulin resistance. Although the concentration of iNOS was found higher in advanced stage NASH than in mild

stage in obese patients with NASH [79], iNOS deficient mice developed NASH by high-fat diet [80]. The issue whether iNOS is harmful in the liver remains unestablished.

Sources of Liver Fat

Accumulation of triglycerides as fat droplets within the cytoplasm of hepatocytes is a prerequisite for subsequent events of NASH. Accumulation of excess triglyceride in hepatocytes is generally the result of increased delivery of non-esterified fatty acids (NEFAs), increased synthesis of NEFAs, or impaired intracellular catabolism of NEFAs, impaired secretion as triglyceride, or a combination of these abnormalities (Figure 3) [1]. Recent techniques such as isotope methodologies, multiple-stable-isotope approach and gas chromatography/mass spectrometry provided valuable information regarding the fate of fatty acids during both fasting and fed states [81] such as the relative contribution of three fatty acid sources to the accumulated fat in NAFLD: adipose tissue, de novo lipogenesis, and dietary (see below). Additionally, these studies reported that plasma NEFA pool is the main contributor of both hepatic-triglycerides in the fasting state and VLDL-triglycerides in both fasting and fed states (see below).

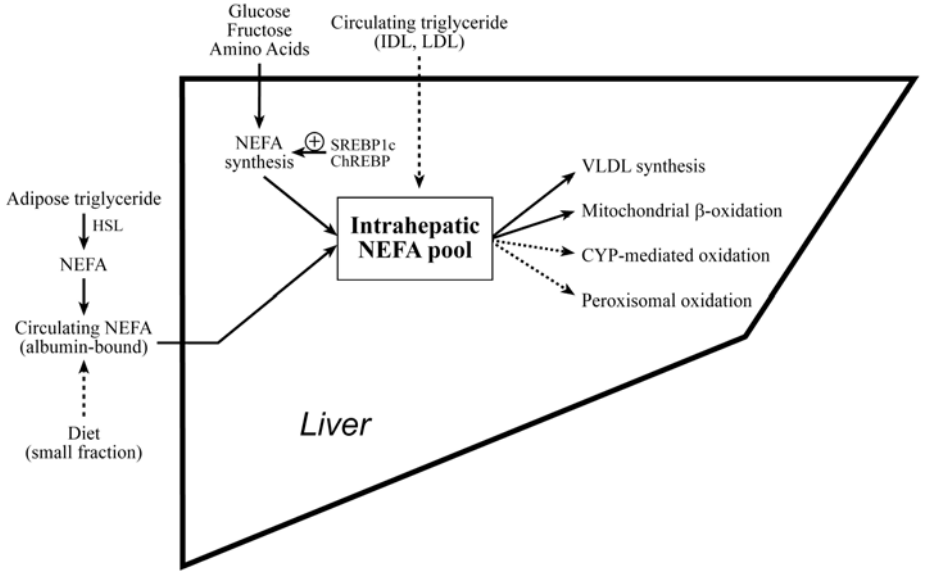

Figure 3. Sources and fates of liver fat. The major sources of fat in the liver are delivery as NEFA from adipose tissue and de novo lipogenesis from carbohydrates and amino acids. Short chain NEFA from the gut are a small fraction of total circulating NEFA in the fed state. Uptake of triglyceride in the form of LDL and IDL constitutes a minor fraction. The intrahepatic NEFA pool has two major fates. Some undergoes mitochondrial β-oxidation while most is generally re-esterified to triglyceride, incorporated into VLDL and secreted into the circulation. Catabolic pathways that contribute to the disposition of a minor fraction of NEFA include peroxisomal β-oxidation and cytochrome P450 mediated ω-oxidation. Although peroxisomal and CYP oxidation is quantitatively small, it may increase the burden of oxidant stress in the liver. NEFA: non-esterified free fatty acids; HSL: hormone sensitive lipase; IDL: intermediate density lipoproteins; LDL; low density lipoproteins; VLDL: very-low-density lipoproteins.

Dysregulated Peripheral Lipolysis

After a meal, insulin normally inhibits peripheral lipolysis by inhibiting hormone sensitive lipase, while reducing β-oxidation of fatty acids and increasing fatty acid synthesis from the glucose in the liver [30,44]. Moreover, under physiologic conditions, insulin inhibits the hepatic secretion of VLDL-triglycerides to the circulation by inducing apoB 100 degradation in the liver [30,82] while increased fatty acid flux into the liver increases hepatic-VLDL synthesis [83]. Additionally, free fatty acid trafficking between the adipose tissue and the liver would not cause accumulation of fatty acids in the liver under physiologic conditions. However, regulation of hormone sensitive lipase is diminished in the insulin resistant states [21,84] and lipoprotein lipase activity in adipose tissue is reduced due to the insulin resistance [30]. Hormone sensitive lipase catalyzes the hydrolytic release and mobilization of fatty acids from the increased adipose tissue triglycerides in obese subjects with insulin resistance. Increased triglyceride lipolysis enhances NEFA burden in the circulation. A recently performed NAFLD study with the combination of recent techniques (see above) showed that adipose tissue makes a major contribution to plasma NEFA pool, contributing 81.7% in fasted state and 61.7% in fed state [81]. Additionally, the contribution of dietary lipids to the plasma NEFA pool was found to be only 26.2% and 10.4% in fed and fasted states respectively in the same study. Finally, the contribution of newly made fatty acids (originating from the adipose tissue and liver) to the plasma NEFA pool was 7.0% and 9.4% for the fasted and fed states, respectively.

The liver takes up free fatty acids from the circulating NEFA pool and the rate of uptake depends only on the plasma free fatty acid concentrations. Hepatic NEFA uptake continues despite increased hepatic content of fatty acids and triglycerides [44,85] and there is no known regulatory mechanism or limitation of this process. The concentration of free fatty acids is increased in the portal circulation rapidly when the lipolysis occurs in visceral adipose tissue [30]. These products directly flux to the liver via the splanchnic circulation and contribute to hepatic triglyceride synthesis, NAFLD, and hepatic insulin resistance. Additionally, decreased adipocyte glucose uptake due to insulin resistance reduces glycerol-3-phosphate concentration in adipose tissue. This diminishes the conversion of fatty acids into intracellular triglyceride and further increases the plasma NEFA pool.

Hepatic de Novo Lipogenesis (DNL)

Hepatic de novo lipogenesis (fatty acid and triglyceride synthesis) is increased in patients with NAFLD. Stable-isotope studies showed that increased DNL in patients with NAFLD contributed to fat accumulation in the liver and the development of NAFLD [81,86]. Specifically, DNL was responsible for 26% of accumulated hepatic triglycerides [81] and 15-23% [81,86] of secreted VLDL triglycerides in patients with NAFLD compared to an estimated less than 5% DNL in healthy subjects and 10% DNL in obese people with hyperinsulinemia [87-89]. Interestingly, Donnelly and colleagues demonstrated the similarity between VLDL-triglycerides and hepatic-triglycerides regarding contributions of fatty acid sources such as 62% vs 59% for NEFA contribution, respectively; 23% vs 26% for DNL, respectively; and 15% vs 15% for dietary fatty acids, respectively in NAFLD patients [81]. These studies also showed that increased DNL in the fasting state is not increased more in fed state.

Substrates used for the synthesis of newly made fatty acids by DNL are primarily glucose, fructose, and amino acids; oleic acid (18:1, a ω-6 monounsaturated fatty acid, which is relatively resistant to peroxidation) is the major end product of de novo fatty acid synthesis. Other studies have shown that oleic acid is one of major fatty acids found in the liver in humans [90] as well as in mice with NAFLD [91]. Oleic acid is also a common dietary fatty acid type. Listenberger and colleagues demonstrated that oleic acid is readily incorporated into triglycerides and leads to the accumulation of triglycerides which was well-tolerated by cultured cells [92]. Moreover, these studies demonstrated that the cellular ability to produce triglycerides from fatty acids is strongly associated with the protection from lipotoxicity. Most importantly, this process appears a cellular adaptation mechanism against changed environmental conditions such as increased fatty acid flux into the liver in obese patients with insulin resistance. However, lipotoxicity might occur over time by chronically increased fatty acid supply when the triglyceride synthesis and storage capacity are exceeded. Palmitic acid, a saturated fatty acid, alone has no ability to incorporate into triglycerides and causes lipoapoptosis by generating both ROS and ceramides. Another crucial observation in these studies is that oleic acid generated endogenously by DNL or exogenously prevents palmitic acid-induced apoptosis. These effects had been achieved by oleic acid-inducing palmitate incorporation into triglycerides. However, lipotoxicity might occur by decreased or overwhelmed triglyceride synthesis capacity, even in oleic acid rich-medium.

Regarding NAFLD, the purpose of the increased oleic acid synthesis by DNL might be a buffer against chronically increased fatty acid supply to the hepatocytes. We might also propose that all fats in the liver might not be harmful, even they might be evidence of a protective mechanism against increased fatty acids. This might be also an explanation for whether mild degree steatosis, less than 5% fat, is important.

Although a growing body of literature suggests that NAFLD is primarily associated with a peripheral insulin resistant state, there is also a relationship between NAFLD and hepatic insulin resistance. Hepatic insulin resistance causes dysregulation of hepatic lipogenesis and fat accumulation within hepatocytes. Moreover, the contribution of hepatic insulin resistance on the development of type 2 diabetes mellitus is critical, with both increased hepatic glucose production and postprandial hyperglycemia [37,93]. One mechanism of hepatic insulin resistance in NAFLD was recently demonstrated in rats in which hepatic fat accumulation was a specific cause of hepatic insulin resistance [55]. After high-fat feeding for 3 days, rats showed increased hepatic fat content (triglycerides and fatty acyl-CoA) which originated from diet, hepatic insulin resistance, blunted insulin-stimulated IRS-1 and IRS-2 tyrosine phosphorylation, increased activation of PKCε and JNK-1, diminished insulin activation of AKT2 and inactivation of GSK3 while there was no significant peripheral insulin resistance, and no significant increase in the fat content of muscle and adipose tissue. In this model, increased hepatocellular fatty acid metabolites activated PKCε and JNK-1 which impaired IRS-1 and IRS-2 tyrosine phosphorylation and subsequently caused hepatic insulin resistance.

Elevated insulin and glucose concentrations in the plasma, abnormalities that characterize insulin resistance, independently stimulate DNL in the liver through activation of hepatic SREBP-1c and carbohydrate response element binding protein (ChREBP), respectively [94]. SREBPs are transcription factors involved in the uptake and synthesis of

fatty acids [75,95-97]. The SREBP family includes SREBP-1a, 1c, and 2. SREBP-1c is predominantly located in the liver and can activate transcriptionally the genes involved in hepatic lipogenesis [75,97]. A study performed with *ob/ob* mice deficient for SREBP-1c demonstrated 50% reduction in hepatic triglyceride content [98]. Fasting reduces and feeding increases the amount of SREBP-1c in the liver. In patients with NAFLD, insulin continues to stimulate SREBP-1c mediated lipogenic genes expression despite profound insulin resistance. SREBP-1c also stimulates the expression of enzymes that produce malonyl-CoA at the mitochondrial membrane, a molecule that potently inhibits mitochondrial fatty acid uptake and β-oxidation. Fatty acids thus undergo triglyceride synthesis or oxidation in peroxisomes and smooth endoplasmic reticulum which produces more ROS. Thus, SREBP-1c activation not only favors the formation of fatty acids, but it also down-regulates their catabolism which further contributes to the formation of triglyceride.

Fatty acid synthesis is only partially (30-50%) dependent on SREBPs [99]. Another transcription factor, ChREBP, regulates the genes involved in the synthesis of fatty acids from glucose [100,101]. Elevated plasma glucose levels stimulate cytoplasmic ChREBP to enter the nucleus and bind to DNA leading to specific gene expression. For example, activated ChREBP activates liver type pyruvate kinase which increases both glycolysis to produce more citrate and stimulate DNL to produce fatty acids.

Uptake of Dietary Fat into the Liver

In the fed state, most triglyceride in the plasma is found in gut-derived chylomicrons or liver-derived VLDL. Only a small fraction of gut-derived triglyceride is taken up by the liver such that only 15% of liver triglyceride originates from dietary triglyceride while the majority originates from adipose-derived NEFA [81]. In the fasted state, triglycerides found in the plasma are primarily remnant lipoproteins such as chylomicron remnants, VLDL remnants, and intermediate density lipoproteins (IDL) [44]. Triglyceride content of remnant molecules differs between healthy and insulin resistant states because hepatic uptake is a direct function of the level of dietary fat intake, rate of hepatic secretion of VLDL, and the activity of adipose lipoprotein lipases. It was shown that high triglyceride content of remnants in insulin resistant subjects increased VLDL synthesis and secretion in both human and cultured liver cells compared to healthy controls. However, remnants were not found to stimulate VLDL secretion from the liver as much as free fatty acids.

These experimental findings are highly relevant to clinical practice. While it may be intuitive to recommend a low fat diet to patients with NAFLD, the benefit of this is primarily in reducing total caloric intake and potentially reducing cardiovascular risks. Moreover, simple sugars have the ability to stimulate lipogenesis [81,88]. Ingested carbohydrates are a major stimulus for hepatic DNL and are thus more likely to directly contribute to NAFLD than dietary fat intake. Additionally, regulation of the changes in hepatic lipogenesis from fasting state to fed state is disturbed.

Moreover, an area of ongoing research is how total caloric intake and the composition of diet affect the development of NAFLD. Studies in alcohol-fed rats showed that polyunsaturated fats are harmful and saturated fats are protective in the liver [102,103]. In contrast, a recently performed study demonstrated that not only polyunsaturated fatty acids, but also saturated fatty acids such as palmitic acid induced hepatocyte apoptosis and injury in

rats [92,104]. Additionally, a low-calorie and very low-fat diet used in one study may have worsened liver inflammation [105]. This observation might be explained by the harmful effect of rapid weight loss or very low fat content of the formula [105,106]. Increased serum concentrations of free fatty acids, which could be due to obesity or rapid weight loss, were also found to be correlated with the severity of fibrosis in patients with NASH [107].

Fates of Liver Fat

Very-low-density Lipoprotein (VLDL) Synthesis and Secretion

VLDL is a lipoprotein complex of apoB 100, triglycerides, cholesteryl esters and phospholipids synthesized only in the liver [44,108-111]. Synthesis occurs in the endoplasmic reticulum and VLDL is exported by vesicular transport from the liver into the plasma. Lipoprotein lipases in the vascular endothelium progressively remove triglyceride from circulating VLDL to produce ILD and smaller VLDL particles. Such delipidated products can be taken up by the liver but constitute a relatively minor pathway of fat uptake in the liver. The relative contributions of fatty acids derived from adipose tissue, diet, and DNL to the triglyceride content of VLDL in fasted and fed states were 60.4% and 27.9% for adipose, respectively; 12.1% and 19.1% for diet, respectively; and 22.2% and 20.4% for DNL, respectively in patients with NAFLD [81]. The similarity between VLDL-triglycerides and hepatic-triglycerides regarding contributions of fatty acid sources was also demonstrated (see above) [81]. The plasma NEFA pool contribution derived from adipose tissue comprised the largest fraction in both fed and fasted states.

Inhibition of VLDL assembly or secretion due to any reason leads to hepatic steatosis. The factors regulating apoB 100 synthesis within the hepatocytes are not completely understood and conflicting data have been reported. ApoB 100 is synthesized and secreted proportional to the amount of available triglyceride in the liver [112,113]. Its synthesis in the endoplasmic reticulum is a rate-determining step for VLDL formation and secretion. This process is facilitated by microsomal triglyceride transfer protein (MTTP) in the lumen of endoplasmic reticulum [114]. Abnormalities of MTTP also have been found to cause hepatic retention of fats and hepatic steatosis. For example, mutations in the promoter and coding regions of the MTTP gene are associated with severe hepatic steatosis and markedly decreased plasma triglyceride levels [115].

Three pathways have been identified for the degradation of this newly synthesized apoB 100 in the liver, namely endoplasmic reticulum associated degradation of newly synthesized apoB 100, reuptake, and postendoplasmic reticulum presecretory proteolysis (PERPP) [110,111,116]. Even though apoB 100 synthesis is regulated, it is synthesized in excess and roughly 70% of newly synthesized apoB 100 is not secreted and undergoes intracellular degradation [117]. The availability of triglycerides for lipidation of apoB 100 is an important factor in preventing apoB 100 from being degraded via the proteasome [116]. PERPP degrades newly synthesized apoB 100, without any contribution of proteasome and lysosomes [116]. Both in vitro and in vivo studies demonstrated that PERPP regulates decreased apoB 100 secretion because of polyunsaturated fatty acids (PUFAs) and increased apoB 100 secretion because of saturated fatty acids [111].

Insulin promotes apoB 100 degradation and decreases hepatic VLDL-triglyceride secretion under physiologic conditions [118]. However, chronic hyperinsulinemia is associated with increased apoB 100 synthesis and increased VLDL-triglyceride concentrations in the circulation, most probably due to resistance to normal insulin action [118-122]. ApoB 100 secretion is increased (40%) in obese and NAFLD subjects, but is significantly decreased (62%) in NASH subjects compared with both obese without NAFLD (body mass index- [BMI], gender-, and age- matched subjects) and lean without NAFLD (age- and sex- matched healthy controls) subjects [109]. Correlated with these findings, the mean metabolic clearance rate of apoB 100 was significantly lower in NASH subjects when compared with both obese without NAFLD and lean without NAFLD subjects. By comparison, the mean absolute synthesis rate of fibrinogen and albumin were not decreased, even significantly increased when compared with lean subjects and similar to that of obese subjects without NAFLD, in NASH in this same study.

One mechanism of impaired VLDL secretion may be increased oxidative stress and lipid peroxidation induced by fatty acids in the liver [111]. Increased hepatic oxidative stress and lipid peroxidation stimulate PERPP to induce apoB 100 degradation and to decrease the secretion of apoB 100, and is associated with lower VLDL concentrations in the plasma [111]. Moreover, lipid peroxidation could achieve these results even in the absence of exogenous fatty acids. It was also reported that feeding rats with PUFAs, which are predisposed lipid peroxidation, led to decreased triglycerides in both the plasma and the liver while hepatic lipid peroxidation products (hepatic lipid hydroperoxides and thiobarbituric acid-reactive substances [TBARSs]) were increased and a lipid antioxidant, vitamin E, levels were decreased [123]. An antioxidant (an iron chelator or a lipid antioxidant) added to the medium decreased oxidative lipid peroxidation, improved apoB 100 concentrations, and increased VLDL-triglyceride secretion in both rat hepatoma and primary rodent hepatocytes [111]. PUFA infusion also increased hepatic lipid peroxidation and decreased hepatic VLDL secretion in mice [111]. These studies also pointed out a direct oxidative damage to apoB 100 via enzymatic or non-enzymatic pathways.

These abnormalities are correlated with the pathogenesis of NASH. Oxidative stress and -related hepatic lipid peroxidation are associated with the development of NASH in both animal models and humans. In addition to increased free fatty acid flux into the hepatocytes, increased oxidative stress and lipid peroxidation are associated with both increased degradation and decreased secretion of apoB 100 induce lipid retention and accumulation in the liver. Moreover, the finding of Charlton and colleagues of decreased apoB 100 synthesis in NASH patients (see above) [109] might be explained by the increased oxidative stress and lipid peroxidation, PERPP degradation, in patients with NASH.

Polymorphisms of the apoB100 gene may also impair VLDL secretion. Several apoB 100 gene mutations have been reported in patients with NAFLD that lead to the synthesis of truncated apoB 100 [124,125]. According to some investigators there are two types of apoB 100 deficiency related with NAFLD, namely absolute deficient type (rare) and relative deficiency (ordinary type) [108,114].

In summary, apoB 100 synthesis and secretion is increased in fatty liver subjects but this process might still not enough for a normal VLDL assembly and triglyceride secretion. This causes the accumulation of triglycerides and eventually NAFLD.

Mitochondrial β-Oxidation

Fatty acids have two major fates in the liver, namely esterification to form triglycerides that are secreted as VLDL and mitochondrial β-oxidation. Mitochondrial β-oxidation of short, medium, and long chain fatty acids involves multiple steps which include entry of long chain fatty acids into the mitochondria, a process dependent on carnitine shuttle enzymes CPT-I (carnitine palmitoyltransferase 1; an outer membrane enzyme) and CPT-II, and the β-oxidation of fatty acids to form progressively shorter acyl-CoA moieties, acetyl-CoA [126]. Then, acetyl-CoA subunits are completely degraded by the tricarboxylic acid cycle to carbon dioxide. These oxidation processes are associated with the reduction of oxidized NAD+ and FAD to NADH and $FADH_2$. Reoxidation of NADH and $FADH_2$ to NAD+ and FAD produces electrons which transfer to the mitochondrial respiratory chain (MRC) [44,126-128]. Most of the electrons of NADH and $FADH_2$ are safely transferred to oxygen to form water in a process that generates ATP through the MRC. Partially reduced oxygen molecules, termed reactive oxygen species or ROS, are constitutively generated during this process when the electrons of NADH and $FADH_2$ directly react with oxygen and may contribute to oxidant stress if endogenous protective mechanisms are overwhelmed [126].

In the fasting state of lean subjects, NEFA are released from adipose tissue, enter into the liver and are rapidly metabolized by mitochondrial β-oxidation as a source of energy. Necessary for this to occur is a state of low hepatic malonyl-CoA concentrations which is a common feature in fasting state. Malonyl-CoA is produced by acetyl-CoA carboxylase which is the first step in fatty acid synthesis. Under physiologic conditions, adipocytes of lean people store lipids after meals and release them during the fasting period [118]. In contrast, heavily lipid-laden adipocytes in obese people continue to release fatty acids in the immediate postprandial term. Consistent with the increased flux of NEFA to the liver in obese patients with NAFLD, mitochondrial β-oxidation of fatty acids in the liver is also increased and as such may contribute to increased generation of ROS and oxidant stress [126]. Although insulin and malonyl-CoA could decrease CPT-I activity in lean people, this effect might be impaired in obese people with insulin resistance.

Excessive fatty acids might use alternative pathways other than mitochondrial β-oxidation to be metabolized and cause mitochondrial injury. These include peroxisomal and cytochrome P450 (microsomal CYP) oxidation systems regulated by mainly fatty acids and insulin [44]. These alternative fatty acid oxidation systems produce more ROS and thus their utilization may be a source of oxidant stress.

Peroxisomal Fatty acid β-Oxidation

One relatively minor fate of fatty acids in the liver is their oxidation in peroxisomes. Peroxisomal oxidation of fatty acids is the normal route of metabolism of very long chain fatty acids (fatty acids with 20 or more carbons) and dicarboxylic acids [44,129]. It might also be involved in the oxidation of fatty acids when mitochondrial β-oxidation is impaired. Peroxisomal oxidation is a four-step pathway in which electrons from the $FADH_2$ and NADH are transferred directly to oxygen. Although this increases the production of H_2O_2, peroxisomes are uniquely endowed with the enzyme catalase that eliminates this reactive oxygen molecule.

Cytochrome P450 Fatty Acid ω (Omega)-Oxidation

Lastly, fatty acids can undergo oxidation by the CYP enzymes of the smooth endoplasmic reticulum which is a relatively minor pathway for the fate of free fatty acids. CYP2E1 and CYP4A isoforms, two such enzymes, are involved in fatty acid oxidation in conditions with substrate overload such as increased free fatty acid concentrations in obesity and increased ketone bodies in type 2 diabetes mellitus. CYP4A upregulation particularly occurs in conditions with decreased CYP2E1 activity. The expression of both CYP2E1 and CYP4A mRNA and their protein levels are increased in both obese and diabetic animal models and humans [130-145]. Their hepatic activity and expression were also reported to be increased in patients with NASH due to the increased substrates, mainly fatty acids and ketone bodies, irrespective of the underlying clinical condition, diabetes or obesity [139,142,143]. The distribution of CYP2E1 is in zone 3 (perivenular) hepatocytes which is the main site of maximal hepatocyte injury in NASH [146]. Nonetheless, the capacity of this enzyme system is very low to handle fatty acids [44,146-148]. Oxidation reactions by the CYP enzymes can be major producers of ROS because of a low degree of coupling between substrate binding and their weak affinity to molecular oxygen, leading to the release of species such as superoxide anion radical, hydroxyl radicals, and hydrogen peroxide.

Peroxisome proliferator-activated receptor-α (PPAR-α), a member of nuclear receptor super family of transcription factors, regulates the genes encoding some mitochondrial and peroxisomal fatty acid β-oxidation enzymes, lipoprotein metabolism, and hepatic fatty acid transport [146,149]. Highly expressed PPAR-α is also involved in hepatocyte proliferation caused by peroxisome proliferators.

Local and Generalized Inflammation in NAFLD

In earlier studies, researchers showed that obesity is associated with low-grade chronic inflammation in both animal models and humans, and this chronic inflammation is a link between obesity and insulin resistance [61,76,150-153]. Insulin resistance is strongly associated with NAFLD. Indeed, several investigators consequently reported that obesity is strongly related with chronic macrophage accumulation within increased adipose tissue in obese mice with high-fat diet-induced or genetically-induced mice [153], and genetically-induced obese mice and human subjects [76]. Xu and colleagues also showed that inflamed macrophages are active within white adipose tissue (WAT) and this activation occurs after increased adiposity and before insulin resistance. The origin of these macrophages might be from the circulation. Macrophages can secrete TNF-α, IL-1, IL-6, and MCP-1. As mentioned previously, these cytokines promote insulin resistance in adipose tissue and eventually increase adipose tissue lipolysis which causes insulin resistance in both muscle and the liver. Weisberg and colleagues also demonstrated that adipose tissue macrophages originating from bone marrow are the major reasons of increased TNF-α expression in adipose tissue, besides significant amount of iNOS and IL-6 expression in both mice and humans [76]. These cytokines and biologically active molecules promote insulin resistance (see above) [68,154-156]. Moreover, the authors reported a positive correlation between adipocyte size and the content (%) of accumulated macrophages in adipose. Additionally, weight loss decreased adipocyte size and improved these metabolic abnormalities [76]. Lastly, Furukawa and colleagues demonstrated increased NADPH oxidase-induced oxidative stress in accumulated

fat of obese mice and humans which promoted dysregulated production of adipocytokines [157]. Increased fatty acids or accumulated macrophages might be the reason of this increased ROS production within adipose tissue. These data indicate localized inflammation and systemic consequences such as insulin resistance and increased circulating free fatty acids. Additional evidence that this chronic inflammation causes insulin resistance comes from the restoring insulin sensitivity by various anti-inflammatory agents such as high dose salicylates via IKK-β inhibiton (see above) or anti-TNF-α antibody infusion [45,63,65].

Loria and colleagues investigated non-organ-specific autoantibodies in patients with NAFLD, and reported that autoantibodies were more prevalent in patients with NAFLD than in general population [158]. Moreover, C-reactive protein levels, as an acute phase protein and inflammation marker, were reported to be elevated in patients with NAFLD and insulin resistant states [159,160]. Lastly, Albano and colleagues investigated circulating IgG antibodies against lipid peroxidation products in 167 patients with NAFLD (79 patients with simple steatosis, 74 with NASH, and 14 with NASH-associated cirrhosis) and compared with 59 age- and sex-matched control subjects [161]. The IgG antibodies were significantly higher in patients with NAFLD than in controls. Additionally, the level and frequency of these antibodies were significantly increased in subjects with advanced fibrosis or cirrhosis, but not increased in patients with steatosis alone or NASH with mild fibrosis. This recent evidence indicates that NAFLD could be the result of generalized inflammation due to oxidative stress and related lipid peroxidation.

NASH: THE PATHOGENESIS OF HEPATOCELLULAR INJURY IN NAFLD

Although much is known about how fat accumulates in the liver, much remains unknown about how this causes sustained hepatocellular injury and the consequences of injury recognized as NASH and fibrosis (Figure 4). Insulin resistance and hyperinsulinemia may contribute to these pathological changes [26]. Chronically increased free fatty acid supply from the lipolytically active adipose tissue to the liver might also contribute to the development of NASH. The prevalence and the severity of NAFLD progressively increase with the number and severity of the features of the metabolic syndrome. Some have argued that the accumulation of fat in the liver is an adaptive change to insulin resistance because of correlates in animals that experience periods of prolonged fasting and intermittent feeding [162]. This argument is correlated with the findings of Listenberger and colleagues that triglyceride synthesis and their accumulation prevented fatty acid-induced lipotoxicity in cultured cells (see above, DNL, oleic acid and comments) [92].

However, the accumulation of fat within the hepatocytes sensitizes the liver to injury from a variety of causes and the regenerative capacity of a fatty liver is impaired [163,164]. These studies also showed that obese mice with fatty liver clear endotoxin less than nonobese controls [163]. This additional stressor is sometimes referred to as a “second hit” in a paradigm that identifies the accumulation of fat as the “first hit” [165]. Possible candidates for the second hit include increased oxidative stress, lipid peroxidation and release of toxic products such as malondialdehyde and 4-hydroxynonenal, decreased antioxidants,

adipocytokines, transforming growth factor-β (TGF-β), Fas ligand, mitochondrial dysfunction, fatty acid oxidation by CYPs (CYP 2E1, 4A10, and 4A14), and peroxisomes, excess iron, small intestinal bacterial overgrowth, and the generation of gut-derived toxins such as lipopolysaccharide and ethanol [1,97,165]. In addition, the regenerative capacity of the fatty liver may be compromised [164,166] and an interacting network of cytokines and adipokines that regulate inflammation is disrupted [167-172].

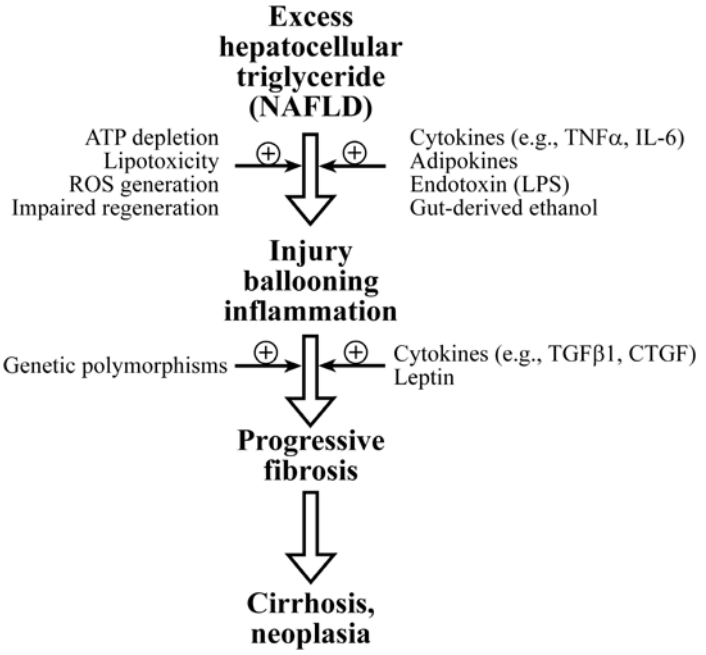

Figure 4. Possible pathway from NAFLD to NASH, cirrhosis and hepatocellular carcinoma. Multiple factors, both within hepatocytes (left side) and extracellular (right side) may contribute to injury of fat-laden hepatocytes, setting in motion the processes that lead to fibrosis, cirrhosis and hepatocellular carcinoma in some patients.

Recently, it was reported that insulin resistance is an independent predictor of advanced fibrosis in patients with NASH [26]. These findings indicate that hypoadiponectinemia, insulin resistance, and high TNF-α concentrations are not only associated with fat accumulation but also contribute to the subsequent injury found in NASH.

Role of Animal Models in Understanding the Pathogenesis of NASH

Understanding the molecular underpinnings of diseases accelerates the development of effective treatment and preventive strategies. Such knowledge can often only be acquired by studies of animal models that recapitulate human disease. Animal models of NAFLD and NASH have been developed and each has its strengths and weaknesses.

The ob/ob Mouse

The leptin-deficient, genetically determined, *ob/ob* mouse becomes both obese and diabetic, and develops NAFLD. This mouse strain exhibits phenotypic similarities to humans with NASH that include insulin resistance, hyperlipidemia, elevated serum TNF-α concentrations, and obesity. This model of murine liver steatosis does not progress to NASH without secondary insults such as lipopolysaccharide (LPS) treatment [173-175]. Deficiency of T-cell mediated immunity due to the lack of leptin might be the reason of these observations [176]. The *ob/ob* mouse shows up-regulated CYP4A and down-regulated CYP2E1 expression [140,177,178]. These observations are interesting because CYP4A upregulation was strongly correlated with the increased prooxidant production in a murine steatohepatitis model (CYP2E1 knockout mice fed MCD diet) (see below) [141]. Additionally, significant hepatic fibrosis may not develop in *ob/ob* mice because of the possible necessity of leptin for hepatic stellate cells (HSC) activation (see below). Furthermore, *ob/ob* mice are relatively protected from cirrhosis. Norepinephrine, a leptin-inducible neurotransmitter, activates HSC appears to be one of the major intermediate signals for this action of leptin which acts via natural killer T (NKT) cells and their products such as IL-10, a profibrogenic cytokine [174,175]. Other genetically determined obese animal models are leptin resistant diabetic (*db/db*) mice and fatty (*fa/fa*) rats.

The Methionine and Choline Deficient (MCD) Diet

One of the animal models used in many studies to further understand the pathophysiology of human NASH, particularly the source of oxidative stress mediators, is rats fed the MCD diet for 4 weeks [138] and mice fed the MCD diet for 10 weeks [141]. The MCD formula includes corn-oil which is largely unsaturated (85%). This kind of fat is an important target of oxidative stress and lipid peroxidation. Although there is a strong histological similarity between this animal model of steatohepatitis and human NASH, MCD diet fed mice are not obese and do not show insulin resistance. On the contrary, MCD diet fed mice have increased insulin hypersensitivity and their serum insulin and glucose levels are lower than wild-type mice fed standard diets (chow fed) [179]. Moreover, these mice lost weight during the experiment despite a relatively higher food intake. However, this kind of nutritional deficiency (MCD) is not common in humans.

MCD diet fed mice have increased total hepatic triglyceride content, steatohepatitis, increased hepatocyte proliferation, decreased circulating triglycerides, elevated liver enzyme levels, overexpression of hepatic CYP2E1 with no significant change in CYP4A isoforms, and increased lipid peroxides which is determined by the measurement of accumulated TBARSs in the liver (about 100-fold increase) [141,180]. Microsomal NADPH-dependent lipid oxidases may also be involved in lipid peroxidation. Mechanisms of injury that might include elevated hepatocellular lipid content which provides a large amount of substrate for lipid peroxidation, inhibition of fatty acid oxidation, induction of CYPs and induction of hepatic lipid peroxidation, could be involved in the development of steatohepatitis of MCD diet fed mice model. The role of TNF-α remains unclear in this murine model of steatohepatitis. There is also no sex hormone associated-effects [180]. PPAR-α deficiency, which causes both mitochondrial and peroxisomal fatty acid β-oxidation defects [180,181],

significantly aggravated pathologic features in the liver (steatosis and steatohepatitis) in MCD diet-fed mice model [180].

Other Dietary Models

Other animal models of steatosis with or without inflammation and fibrosis have been developed by feeding mice a diet with high fat or sucrose or both with or without high caloric intake [144,145,173]. However, the type and the amount of fat of these diets have been highly variable, making comparisons difficult. Moreover, variable amounts of daily caloric intake were allowed by the investigators. A recently described rat model of feeding high-fat liquid diet (71% of energy from fat which included corn, olive, and safflower oil) for 3 weeks was reported as resemble human NASH [144]. These Sprague-Dawley rats exhibited many of the features of human NASH that included obesity, insulin resistance, hyperinsulinemia, increased hepatic TNF-α mRNA expression, induced CYP2E1 and increased CYP2E1 mRNA expression, morphologically abnormal mitochondria, increased both oxidative stress and lipid peroxidation, fatty liver, patchy inflammation, and increased collagen in the liver.

Deng and colleagues recently reported a new murine steatohepatitis model by intragastric overfeeding of male C57BL/6 mice with high-fat liquid diet for 9 weeks [145]. This formula included 37% calories from fat (corn-oil). Of the 13 mice examined, 46% had NASH features. This model showed obesity, increased WAT, insulin resistance, increased serum glucose and leptin concentrations, increased transcription of hepatic lipogenic enzymes such as PPAR-γ, LXR-α (liver X receptor- α) and SREBP-1c, decreased expression of hepatic PPAR-α, induced hepatic CYP4A with down-regulated CYP2E1, increased cytochrome reductase activity, increased hepatic mRNA expressions of TNF-α, IL-1β, IL-6, and MIP-2. These studies also reported, in WAT, increased inflammation, increased expression of both TNF-α and leptin mRNA, and decreased expression of adiponectin mRNA.

Transition from Simple Steatosis and NASH to NASH-Associated HCC: A new Murine NASH-Associated Hepatic Neoplasia Model

Xu and colleagues, recently developed a murine NASH-associated hepatic neoplasia model with the somatic inactivation of the Nrf1 gene in the livers of adult mice [182]. The authors reported that liver specific Nrf1 gene deficient mice showed similar sequence of events and the progression to histological features of human NASH. Decreased expression of antioxidant response elements containing genes and upregulation of CYP4A genes were also demonstrated. This murine hepatic neoplasia model had evidence of increased oxidative stress with the proliferation of endoplasmic reticulum before the development of liver cancer. Sustained oxidative injury and its consequences with activated hepatocyte proliferation may increase the possibility of liver cancer development in these mutant livers. This and similar models may play an important role in the further understanding of the pathophysiology of NASH and its consequences.

Oxidative Stress and the Pathogenesis of NASH

A logical and attractive hypothesis is that oxidative stress in triglyceride-loaded hepatocytes is the cause of sustained injury with consequent NASH, fibrosis and cirrhosis [1,165,183]. The imbalance between the increased ROS and decreased antioxidants leads to lipid peroxidation of PUFAs, cellular membranes, mitochondrial membranes, and DNA [21,146,184-187]. ROS have relatively short-lived and local effects while lipid peroxidation products have longer half-lives and the capability to reach extracellular targets. Lipid peroxidation produces cytotoxic aldehydes such as malondialdehyde and 4-hydroxynonenal. ROS and these aldehydes further contribute to oxidative stress, decreased ATP production, and increased proinflammatory cytokine release. These events promote hepatocyte injury, necroinflammation, hepatocytes apoptosis, and fibrosis. Hepatocyte ballooning and the development of megamitochondria with true crystalline inclusions (MMC) might be the result of this oxidative stress and lipid peroxidation as well.

Despite the attractiveness of this hypothesis, supporting data has been sparse. Some studies have suggested a benefit of the antioxidant vitamin E [188-190], but effective antioxidants have not been rigorously tested in clinical trials. Most clinical studies only provide correlations between the presence of NASH and elevated indices of oxidant stress without establishing a causal relationship [21,146,184,187,191]. Additionally, the lipid peroxidation product 4-hydroxynonenal was found more in perivenular zone (zone 3) than periportal zone in patients with NASH, correlating with the histological lesions of NASH that are predominantly in zone 3 [187]. Moreover, more evidence of lipid peroxidation and oxidative DNA damage has been found in NASH than in simple steatosis. Lipid peroxidation was greater in patients with NASH than in patients with simple steatosis. The same study also showed that increased 4-hydroxynonenal strongly correlated with both the grade of necroinflammation and the stage of NASH, but not with the grade of steatosis while increased evidence of oxidant damage to DNA as measured by 8-hydroxydeoxyguanosine only correlated with the grade of necroinflammation in patients with NASH. This being said, oxidant stress could play a central role in causing NASH and our clinically available antioxidants may simply be ineffective at preventing the disease to prove the point. A number of sources of increased ROS production have been established in NASH that include proinflammatory cytokines such as TNF-α, iron overload, overburdened and dysfunctional mitochondria, CYPs, and peroxisomes.

Mitochondria as a Source of Oxidant Stress

The hepatocyte mitochondria are the main site of β-oxidation of free fatty acids. The electrons removed from free fatty acids during β-oxidation are shuttled through the mitochondrial electron transport chain (MRC), eventually leading to ATP synthesis and the generation of carbon dioxide and water (see above). Inherent in this process is the dissociation of partially reduced molecular oxygen in the form of superoxide, hydrogen peroxide and the hydroxyl radical, species collectively termed reactive oxygen species, or ROS. About 1%-5% of oxygen consumed during cellular respiration is not fully reduced to water during this process under physiologic conditions [192] and the production of these

ROS is further increased in dysfunctional mitochondria. Thus, mitochondria have been proposed to play a central role in the pathogenesis of NASH [126].

Mitochondria also increase their oxidation capacity for the increased fatty acid flux as observed in obesity and insulin resistant states in humans and in animals fed high-fat diet. However, this increase has its limits and excess free fatty acids are metabolized at other sites in hepatocytes such as peroxisomes (β-oxidation) and the smooth endoplasmic reticulum (ω-oxidation). Acyl-CoA oxidase (AOX) catalyzes the initial reaction of fatty acid oxidation in peroxisomes, a process that generates hydrogen peroxide and thus may contribute to oxidant stress.

P450 as a Source of Oxidant Stress

Fatty acids not oxidized by mitochondria are mainly oxidized by CYP2E1, a process that further increases ROS production within the hepatocytes [146,193,194]. Other CYP isoforms that may generate oxidant stress include CYP4A family such as CYP4A10 and CYP4A14, which is less active than CYP2E1 and mainly active in the setting of low concentration or deficiency of CYP2E1 [141]. The major function of this enzyme system is to metabolize endogenous lipophilic substrates such as steroid hormones, lipophilic xenobiotics, drugs and other environmental toxins. Moreover, CYPs could metabolize and activate carcinogens. Increased endogenous substrate burden such as increased levels of free fatty acids (e.g., due to increased peripheral lipolysis in obesity) and ketone bodies (increased in diabetes) induce CYP2E1 expression in humans [139,142,143,195,196].

In normal conditions, CYP2E1 oxidation produces oxygen radicals, but the balance between these ROS and the abundance of endogenous antioxidants determines the extent of resulting oxidant stress. Initial studies demonstrated increased CYP2E1 expression in diabetic or obese rats fed a high-fat diet [132,133,136,144,145,197,198] as well as in rats and mice fed a MCD diet [138,141]. Later evidence demonstrated increased hepatic CYP2E1 expression by immunostaining of paraffin-embedded liver biopsy sections in patients with NASH [139]. In contrast, hepatic content of CYP3A was decreased in all liver sections from patients with NASH. The same study additionally showed that zone 3 steatosis, which is the typical acinar localization in NAFLD, was closely associated with increased CYP2E1 expression and in some cases extending into zones 2 and 1. CYP2E1 activity was also found to be significantly higher in nondiabetic patients with NASH than healthy controls matched for sex, BMI, and age [142]. The authors assessed the hepatic CYP2E1 activity with oral clearance of chlorzoxazone, a potent skeletal muscle relaxant and in vivo CYP2E1 probe, in this study. Only nocturnal hypoxemia and β-OH butyrate were the independent predictors of increased hepatic CYP2E1 activity. In the same study, a significant increase in the lymphocyte CYP2E1 mRNA expression was demonstrated in the NASH cohort while there was no significant correlation between increased lymphocyte CYP2E1 mRNA expression and hepatic CYP2E1 activity [196]. Increased fasting insulin and insulin resistance were shown in a nondiabetic NASH cohort while fasting glucose levels did not significantly differ from the healthy controls (see below; insulin up-regulated the expression and the activity of hepatic CYP2E1 in primary cultured rat hepatocytes). Another study reported a positive correlation between the severity of hepatic steatosis and hepatic CYP2E1 activity by the oral clearance of chlorzoxazone in morbidly obese patients with NASH [143]. These studies also showed

that weight loss decreased hepatic CYP2E1 activity. In addition to the activation of CYP2E1, there are two other cytochrome P450s, namely CYP4A10 and 4A14, that have been suggested to play a role in animal studies (see above) [141]. CYP4A family is induced by PPARα that PPARα-deficient mice prevented the development of NASH.

Several investigators previously reported that increased mitochondrial and peroxisomal β-oxidation of fatty acids provided a large amount of ketone bodies to hepatic cytochromes. This induces cytochrome P450 gene expression and increases their protein level in the liver. However, this issue remains controversial with some recent observations. Woodcroft and colleagues used primary cultured rat hepatocytes in the absence of insulin to evaluate the effect of increased ketone bodies on the regulation of CYP2E1 expression, and showed no effect or even decreased CYP2E1 mRNA levels [199]. Moreover, these studies demonstrated that insulin decreased CYP2E1 mRNA and its protein levels by both suppressing CYP2E1 gene transcription and enhancing CYP2E1 mRNA degradation in an increased insulin concentration-dependent manner [199-201]. Similarly, De Waziers and colleagues previously had reported increased degradation of CYP2E1 mRNA by insulin in Fao rat hepatoma cells [202]. Additionally, Favreau and colleagues had demonstrated that administration of insulin reversed the increased expression of CYP2E1 in rats [132]. Wang and colleagues showed insulin supplementation in type 1 diabetics achieved close to normal CYP2E1 activities (similar to healthy controls) [196]. Furthermore, Woodcroft and colleagues reported that increased concentration of glucose in the medium might elevate CYP2E1 mRNA levels [199]. In parallel, Leclercq and colleagues previously had reported that dietary sugar restriction decreased CYP2E1 activity in human [203]. Lastly, Wang and colleagues showed an inverse relationship between chlorzoxazone area under the curve and fasting glucose levels [196]. These novel studies pointed out that insulin rather than ketone bodies, with or without glucose contribution, regulates the expression and activity of hepatic CYP2E1. With respect to the pathogenesis of NAFLD, insulin resistance and hyperglycemia are major metabolic hallmarks of NAFLD. These metabolic abnormalities increase hepatic CYP2E1 activity and subsequent prooxidant production in patients with NAFLD.

Moreover, Nieto and colleagues reported that CYP2E1-mediated oxidative stress induced collagen type 1 expression in rat HSC [204]. However, CYP2E1 expression was not demonstrated in human HSC [205]. It is also well-defined that CYPs both metabolize and activate carcinogens. It might be possible that increased production of activated carcinogens by CYPs might contribute to the development of liver cancer in patients with NASH.

Iron, Oxidant Stress and NASH

Iron can play a central role in promoting oxidant stress and this is proposed to be the mechanism of progressive liver disease in hemochromatosis. However, there is no convincing evidence for the role of iron in the pathogenesis of NASH [206-209]. Plasma and hepatic iron measurements, plasma ferritin levels, and genetic mutations of hemochromatosis gene (*HFE*) are the main parameters which have been used to investigate the contribution of iron in the pathogenesis of NASH. Recently, a large-population based study reported a correlation between elevated serum alanine aminotransferase levels and increased serum transferrin and iron concentrations [210]. Antioxidants were decreased as well. Another recent study evaluated 42 patients with carbohydrate-intolerance who had serum iron saturation lower

than 50% and no C282Y and H63D *HFE* mutations [211]. After initial measurements, investigators induced iron depletion to a level of near-iron deficiency by phlebotomies. Interestingly, they observed improvements in both insulin sensitivity and serum alanine aminotransferase activity in some of the patients, indicating that iron may play a role not only in oxidant stress but also in the initial predisposing factor of insulin resistance. A recent prospective cohort study evaluated 263 patients with NASH for both hepatic and peripheral iron burden and *HFE* mutations (C282Y and H63D) and the investigators found that iron burden and *HFE* mutations did not significantly correlate with the hepatic fibrosis of NASH [26].

Mitochondrial Dysfunction and ATP Depletion

Mitochondria are the organelles primarily responsible for fatty acid β-oxidation and oxidative phosphorylation, the process responsible for the production of ATP. Mitochondria are also a source of a limited amount of ROS production under physiologic conditions (see above) [126,128]. Several observations including decreased mitochondrial enzyme activities and increased fat concentration of skeletal muscle cells in obese or diabetic patients have suggested mitochondrial dysfunction in these disorders. Such abnormalities may increase ROS production and promote both oxidative stress and lipid peroxidation within the hepatocyte. Mitochondrial dysfunction is frequently due to a combination of genetic abnormalities, physical inactivity, aging, lipotoxicity (free fatty acids), lipid peroxidation (mitochondrial DNA alterations), and TNF-α [118,126].

The hepatocyte is a cell rich in mitochondria and some studies have suggested that each hepatocyte contains approximately 800 mitochondria, although other investigators have suggested that mitochondria form an interconnected network and are thus difficult to enumerate [127,128,162]. Mitochondria contain their own genomic DNA located in the matrix and this DNA encodes a limited number of components of the MRC. The majority of mitochondrial proteins are encoded by nuclear DNA. Hepatic mitochondrial abnormalities have been identified in NAFLD, suggesting that mitochondria may be the source or target of injury and that ineffective mitochondrial function resulting in cellular ATP depletion may be important pathophysiological processes in NAFLD and NASH [212]

The presence of megamitochondria, or mitochondrial swelling, is a microscopically detectable structural abnormality of hepatocyte mitochondria found in a variety of liver diseases including NAFLD [21,213,214]. Crystalline inclusions within the mitochondrial matrix have been documented in patients with NASH by electron microscopy. The composition and function of these crystals remain to be established. The presence of megamitochondria might be related to MRC enzyme complex deficiencies or oxidative phosphorylation abnormalities of mitochondria. In one study, the presence of lipid peroxidation, demonstrated by 3-nitrotyrosine staining in liver specimens, was noted to a minor degree in normal livers and was marked in both fatty liver and NASH with significantly higher amount in NASH than in fatty liver [21]. The same study also showed that the abundance of megamitochondria with crystalline inclusions was increased in patients with NASH (nine of ten patients) compared to patients with steatosis alone (none of eight

patients), hepatitis C (one of ten patients), and controls (none of six potential donors). Marked differences in mitochondrial inclusions within the same liver and cell to cell variability for this feature in patients with NASH were also noted [21,213,215]. Despite the correlation of mitochondrial abnormalities with NASH, another study of NASH patients reported that there was no correlation between the abundance of megamitochondria and the stage of NASH (stages 1 and 2 vs stages 3 and 4), zones of NASH (zone 1 vs zone 3), severity of lipid peroxidation (low vs high), and ballooning hepatocytes (0-1 vs 2-3) [214]. These studies have also found that two patients with NASH-associated cirrhosis lose their mitochondrial inclusions as well as other histologic features of NASH by the time their disease has progressed to cirrhosis [10,214,216]. Why this occurs has not been established.

Hepatic mitochondrial DNA levels and the protein products of the mitochondrial genes are also decreased in patients with NASH. Earlier studies reported normal activity of complex I and complex III in platelet-derived mitochondria of patients with NASH [213], although no defect in the MRC enzyme expression in the muscles of one NASH patient was reported [21]. However, later evidence showed that NASH was associated with decreased cytochrome c oxidase activity in the mitochondria. Finally, decreased hepatic activity of all MRC enzyme complexes by 30% to 50% of control activity (from complex 1 to complex 5) was reported in patients with NASH [217]. Impaired hepatic MRC function increases ROS production and if ROS production exceeds antioxidant capabilities, oxidative stress and injury, lipid peroxidation of macromolecules and cellular membranes, mitochondrial DNA damage, direct damage of several mitochondrial enzymes, and further MRC dysfunction with more prooxidant production are observed. A very recent study pointed out the relationship between long chain fatty acid oxidation abnormalities due to a mitochondrial trifunctional protein (MTP) defect and the development of both insulin resistance and hepatic steatosis in mice [218]. In addition to a MTP defect, aging was an important factor in the development of these disturbances. Mixed macro- and microvesicular steatosis due to β-oxidation defects in the mitochondria was the predominant type of steatosis in this study and CYP 2E1 expression was upregulated and levels of the antioxidant glutathione were decreased.

TNF-α, a cytokine implicated in NASH, diminishes hepatocyte mitochondrial permeability, blocks MRC electron flow, and eventually causes increased ROS production [126,167,217, 219]. A study recently demonstrated a significant correlation between increased circulating TNF-α levels and mitochondrial dysfunction in patients with NASH [217].

Mitochondrial uncoupling protein 2 (UCP2) is a mitochondrial inner membrane protein. It might regulate proton leak across the mitochondrial inner membrane, promote ATP depletion, and inversely regulate ROS production. Depletion of the energy (ATP) stores increases the susceptibility of hepatocytes to various injury [164] while decreased ROS production limits the hepatocyte injury. Thus, whether UCP2 is harmful or protective in the liver remains unestablished. Several studies demonstrated up-regulation of hepatic UCP2 expression in obese animals provided by genetically (*ob/ob*) or a high-fat diet [164,220-222]. UCP 2 might be responsible for hepatocellular injury in NAFLD, but a recent animal study, performed with UCP2 deficient mice, failed to show any protective or harmful effects of UCP2 in obesity induced fatty livers [223].

Carnitine and two CPTs (CPT-I and CPT-II) are required to transfer long-chain free fatty acids into the mitochondria for β-oxidation. Some investigators reported the role of carnitine deficiency in NAFLD development [224,225] while others observed normal hepatic content of total and free carnitine in patients with NASH [217]. CPT activities were also observed to be normal in patients with NASH [217].

Free Fatty Acid Toxicity

In addition to insulin resistance and hyperinsulinemia, obesity and type 2 diabetes mellitus are strongly associated with increased concentration of free fatty acids in the circulation [64,226,227]. Similar observations have been made in patients with NAFLD [1]. Fatty acids are involved in many important cellular events such as synthesis of cellular membranes, energy storage, and intracellular signaling pathways. However, chronically elevated free fatty acids have the capability to disturb diverse metabolic pathways and induce insulin resistance in many organ systems (see above, cellular mechanisms of insulin resistance) [107,228-233]. Fatty acids also interact with glucose metabolism. In addition to their metabolic effects, fatty acids could induce cellular apoptosis, also called as lipotoxicity, in two ways: direct toxicity and an indirect effect. One proposed mechanism of fatty acid toxicity in hepatocytes is that fatty acids induce translocation of Bax (which is a mitochondrial protein and a member of Bcl-2 family) to lysosomes and cause lysosomal destabilization which promotes the release of cathepsin B (ctsb, a specific lysosomal enzyme), from lysosomes to cytosol. Subsequently, a cathepsin B dependent process induces NF-κB activation and TNF-α overexpression in the liver [219]. TNF-α might further increase lysosomal destabilization and cathepsin B dependent hepatocyte apoptosis [104,234,235]. Then, cytochrome c release from the mitochondria with mitochondrial dysfunction may occur. Mitochondrial dysfunction causes energy depletion which activates proteolytic caspases and induces DNA fragmentation and chromatin condensation. Moreover, activated caspases cleave the Bcl-2 family proteins and cause further mitochondrial damage while activating DNases that produce DNA breaks [236-238]. NF-κB is a transcriptional factor and has both apoptotic and anti-apoptotic effect. In healthy hepatocytes, activation of NF-κB by TNF-α induces Bcl-2 synthesis which prevents the release of cytochrome c from the mitochondria and subsequent apoptosis [104,239]. Feldstein and colleagues demonstrated that genetically cathepsin B deficient or pharmacologically cathepsin B inactivated mice did not exhibit the development of fatty liver, liver injury, and insulin resistance in a dietary murine model [219]. Moreover, while cathepsin B was demonstrated in hepatocyte lysosomes of healthy control subjects, the majority of hepatocytes in patients with NAFLD showed diffuse distribution of cathepsin B in the cytosol, with a positive correlation with the stage of NASH.

Most recently, Ji and colleagues demonstrated hepatocyte apoptosis induced by the saturated fatty acid palmitic acid in rat hepatocytes [104]. The authors suggested that a mitochondria-mediated apoptosis pathway (intrinsic pathway), which includes two mitochondrial proteins such as Bax and Bcl-2, regulates this process. The authors observed a mild decrease in Bcl-2 levels and a marked increase in Bax levels. Bax induces and Bcl-2

inhibits hepatocyte apoptosis, and they work independently [104,240-242]. The Bcl-2/Bax ratio regulates the release of cytochrome c from the mitochondria and subsequent apoptosis. A significantly decreased Bcl-2/Bax ratio promoted apoptosis in HepG2 cells in these studies [104]. These studies also showed dose- and time-dependent inhibition of cellular growth in rat hepatocytes.

In addition to these mechanisms, there are two other possibilities: ceramide, synthesized de novo from fatty acids and a lipid signaling molecule, might promote apoptosis and elevated free fatty acids may increase oxidative stress and subsequently promote apoptosis [243].

Endogenous Toxins: Endotoxin and Gut-Derived Ethanol

The link between gut flora and liver disease was firmly established after the development of severe and sometimes fatal fatty liver disease in patients with morbid obesity following jejunoileal bypass operation [244]. Some of these patients required liver transplantation and some of the newly transplanted livers developed NASH. It was also observed that antibiotic administration, particularly metronidazole, or surgical removal of the blind loop improved hepatic abnormalities [245-247]. Subsequent observations also include a patient with jejunal diverticulosis and intestinal bacterial overgrowth that appeared to cause NASH [248].

Additional information regarding this process was obtained by animal studies. Investigators showed that *ob/ob* leptin deficient mice produce increased levels of breath ethanol compared to control animals and administration of nonabsorbable antibiotics decreased breath ethanol levels, implicating gut flora as a source of absorbed ethanol in mice [249], a finding not confirmed in humans. A small pilot study performed with obese female patients with NAFLD showed increased breath ethanol concentrations [250]. A subsequent study evaluated the relationship between small intestinal bacterial overgrowth and NASH by measuring a combined ^{14}C-D-xylose and lactulose breath test and correlating these with plasma TNF-α and endotoxin concentrations [251]. Additionally, intestinal permeability was assessed. This study found significantly increased blood TNF-α concentrations and small intestinal bacterial overgrowth in patients with NASH compared to sex and age matched controls. Intestinal permeability and serum endotoxin levels were not different between the groups. However, mean BMI and the prevalence of diabetes were higher in NASH group than controls in this study, suggesting an interplay between insulin resistance and gut-derived endotoxin to cause NASH. The same may be true for gut-derived ethanol as breath ethanol concentrations correlated with increased BMI in NASH patients [250]. The mechanisms underlying these interactions have not been established, but one explanation is increased ethanol and LPS production by bacteria in the small bowel disrupts mucosal integrity and increase intestinal permeability. Absorbed bacterial products may stimulate hepatocytes and Kupffer cells to produce ROS and inflammatory cytokines that contribute to insulin resistance, hepatocyte apoptosis, necroinflammation, and fibrosis. Limited clinical studies have tested this interaction and have found that antibiotics, probiotics, TNF-α receptor antagonism, and surgical elimination of blind loops improved some features of NASH in both animal models and humans [249,252-254].

Adipocytokines

Adipocytokines, adipose tissue derived hormones and cytokines originating from adipose tissue, are often abnormally expressed in patients with NASH and these abnormalities may play a role in pathogenesis of NASH [255-257]. It is now recognized that adipose tissue is not only a storage site for excess metabolic energy in the form of fat, it has also important endocrine and immunologic functions [42,258,259]. Adipose tissue releases a variety of adipocytokines, signaling proteins, fatty acids, and other bioactive lipids that regulate inflammation and metabolism in the liver and elsewhere in the body. Some of the important adipocytokines are TNF-α, IL-6, adiponectin, leptin, and resistin. These adipose tissue products regulate both glucose and lipid metabolisms and insulin sensitivity of the insulin target cells. Additionally, receptors for proinflammatory cytokines such as TNF-α and IL-6 are expressed on the surface of adipocytes indicating that adipocytes, like other insulin-sensitive cells respond to signaling by these mediators. Some adipocytokines such as TNF-α and IL-6 are also the products of macrophages within adipose tissue, a recent finding that suggests an inflammatory state with adipose tissue may regulate metabolism in adipocytes and, by implication, also in downstream tissues such as the liver [76,153]. Furthermore, preadipocytes under some conditions could exhibit phagocytic properties.

The anatomical location of adipose tissue plays an important role in provoking insulin resistance. Visceral, or intraabdominal, fat is lipolytically more active than subcutaneous fat and adipocytes of the former are less mature than those of the latter [260-264]. Visceral adipose tissue is a much more significant source for adipocytokines compared to subcutaneous fat, secreting more TNF-α and leptin while releasing more fatty acids than subcutaneous adipose tissue. In contrast, subcutaneous fat produces more adiponectin than visceral fat. Because of its anatomical location in the mesenteric circulation, visceral adipose tissue releases its adipocytokines and fatty acids directly to the liver via splanchics, a factor that may predispose to NAFLD and NASH. Indeed, removal of subcutaneous fat by liposuction did not improve metabolic abnormalities in one study [265].

Leptin

Leptin is a 16-kDa polypeptide synthesized and secreted by mature adipocytes under the control of *ob* gene [43,266,267]. Skeletal muscle cells and culture-activated HSC might also synthesize leptin and its expression is regulated by IL-1, TNF-α, and insulin [268,269]. Leptin is an endogenous anti-obesity cytokine-type hormone that inhibits food intake and increases energy expenditure at a central level. It has both peripheral actions via the long form of the leptin receptor and central actions via the sympathetic nervous system. The hypothalamus is one of the important sites of leptin effects [270]. Leptin binds the transmembrane leptin receptor Ob-R and Ob-Rb, a long-form leptin receptor, can activate the Janus kinase (JAK)/STAT pathway and phosphorylates STAT proteins [271-274] to induce the transcription of TGF-β1 and procollagen genes. Similarly, leptin causes phosphorylation of STAT-3 in cultured hepatic stellate cells, the cells responsible for fibrogenesis and cirrhosis. However, there is currently no consensus regarding the contribution of leptin to the liver injury and fibrosis [26,168,269,274-280].

As might be expected based on the biological effects of leptin, complete leptin deficient *ob/ob* mice exhibit hyperphagia, obesity, and diabetes caused by a natural homozygous mutation of the *ob* gene [270]. Exogenous leptin administration improved these abnormalities and reduced adipose tissue mass in *ob/ob* mice [43,281-283]. In fact, the beneficial effects of leptin on hyperglycemia and hyperinsulinemia were found with leptin doses which did not induce weight loss [43]. Although leptin may improve insulin sensitivity, the mechanism of this action is not clearly understood. Subjects with generalized lipodystrophy have decreased or absent adipose tissue and low plasma levels of its product, leptin. Loss of adipose tissue causes ectopic adipogenesis such as in the liver and induces insulin resistance in these organs by disturbing downstream insulin signaling. Exogenous leptin administration [284] or implantation of adipose tissue from wild-type mice to mice with generalized lipodystrophy [285] improved metabolic abnormalities such as insulin sensitivity. Improvements in the surgical group were observed after the enlargement and maturation of transplanted adipose tissue.

Leptin also has the ability to regulate immunologic functions such as stimulation of monocytes and induction of TNF-α secretion [176,286-291]. Additionally, leptin might cause oxidative stress, and proinflammatory and profibrogenic processes in the liver. Antisteatotic effects of leptin have been demonstrated in rodents [168,292] while some investigators reported a positive correlation between plasma leptin levels and hepatic steatosis in NASH patients [168]. In contrast, no correlation between serum leptin levels and steatosis, inflammation, ballooning cells, and Mallory bodies was reported [280]. Most recently, Javor and colleagues showed that exogenous leptin administration had no effect on fibrosis stage of NASH patients with severe lipodystrophy. However, the biopsy interval may have been too short to identify differences in this study as the mean duration was only 6.6 months [293].

Patients with absolute leptin deficiency due to a mutation of leptin gene are reported rarely [294]. These patients are morbidly obese and show both insulin resistance and hepatic steatosis. Recombinant methionyl human leptin (r-metHuLeptin) replacement therapy improved NASH activity scores, hepatic steatosis, aminotransferase levels, high triglycerides, fasting glucose levels, insulin resistance, and normalized body weight in leptin deficient, lipodystrophic human subjects [291,293]. This benefit might be related with the inhibition of neuropeptide Y and agouti-related protein synthesis and secretion in the hypothalamus. Other possibilities might be the activation of fatty acid oxidation enzymes, inhibition of lipogenic enzymes, induction of hepatic and adipose tissue PPAR-γ coactivator 1α expression, and activation of PPARα and AMP-activated protein kinase. Leptin might also regulate mitochondrial functions. It was reported that leptin reduced fat content in adipocytes and increased the number of mitochondria while leptin deficiency caused increased fat accumulation in adipocytes and functional deficiencies in the mitochondria [293].

Mutations and truncated leptin receptors have also been reported in humans [43,295]. These patients are obese due to the impaired leptin action. The presence of leptin resistance is also caused by abnormalities of intracellular signaling pathways of leptin. Most obese humans have increased plasma leptin levels which are correlated with adipose tissue mass [296-299]. Weight loss decreased both circulating leptin and inflammation markers [300,301].

Adiponectin

Adiponectin is a large 30 kDa polypeptide hormone (ACRP30) secreted by adipocytes. It has antilipogenic and anti-inflammatory effects [30,257,302-304]. Most evidence suggests that adiponectin is a necessary component of normal insulin action and improves insulin sensitivity by enhancing intracellular insulin signaling [169,305-307], although the adiponectin knockout mice may have normal insulin signaling and glucose tolerance [308]. An interesting relationship has emerged between TNF-α and adiponectin in which each down-regulates the expression and activity of the other [309-311].

At the cellular level, adiponectin induces β-oxidation of fatty acids and decreases muscle steatosis. Adiponectin decreases fatty acid content of the liver and increases hepatic insulin sensitivity by decreasing both plasma free fatty acid uptake and de novo synthesis of fatty acids and by increasing both mitochondrial β-oxidation of fatty acids and triglyceride export [302,312,313]. These effects reduce triglyceride content and glucose output of the liver. Adiponectin also may activate AMP-activated protein kinase and directly stimulate glucose uptake in both adipocytes and muscle cells. In addition to these effects, adiponectin may have anti-inflammatory properties such as inhibition of both phagocytic activity and TNF-α production of macrophages [314,315].

There is an inverse relationship between adiponectin mRNA expression and adipose tissue mass in both mice and humans. Plasma levels of adiponectin were also found to be inversely related to the adipose tissue mass and degree of insulin resistance in human subjects [316-318]. A study performed in Pima Indians showed that increased plasma adiponectin levels strongly correlated with a decreased risk of developing type 2 diabetes mellitus, independent of the presence of obesity [319]. Plasma adiponectin levels are inversely correlated with hyperinsulinemia and insulin resistance. This inverse relationship is less marked with increased adipose tissue mass. In addition to an increase in inflammatory response, adiponectin knockout mice also have high plasma levels of TNF-α and severe insulin resistance [169,305]. As might be expected, lipoatrophic mice that lack normal adipose tissue show decreased plasma adiponectin levels, as well as leptin deficiency and insulin resistance. These abnormalities could be reversed with the adiponectin administration.

Leptin-deficient *ob/ob* mice have reduced adiponectin concentrations and adiponectin treatment improved hepatomegaly and steatosis and decreased elevated serum aminotransferases and inflammation of the liver by inhibiting hepatic TNF-α production and fatty acid synthesis, and increasing fatty acid oxidation [170]. Adiponectin administration prevented hepatic fibrosis in wild-type mice treated with carbon tetrachloride. Moreover, the same study also demonstrated aggravated liver fibrosis in adiponectin knockout mice treated with carbon tetrachloride. Although patients with NASH have excess visceral fat, circulating adiponectin concentrations were found decreased independent of insulin resistance [171,320,321]. An association between reduced adiponectin levels and more extensive hepatic necroinflammation was also demonstrated [171]. Two adiponectin receptors, defined as AdipoR1 and AdipoR2, are expressed mainly in skeletal muscle and liver, respectively [302]. AdipoR1 has a high affinity for circulating globular adiponectin (gAd) while AdipoR2 has an intermediate affinity for both forms of adiponectin, full-length ligand and gAd. The levels of hepatic AdipoR2 mRNA expression in patients with NASH is uncertain because of conflicting data [320,321]. Thus, it remains unclear whether decreased hepatic Adipo R2 is

an adaptive mechanism against decreased circulating adiponectin concentrations in patients with NASH.

TNF-α

TNF-α is a proinflammatory cytokine primarily synthesized and secreted by adipose tissue in the absence of malignancy or infection [30,43,322,323]. In addition to inflammation, TNF-α is involved in cell proliferation, differentiation, and apoptosis. Increased TNF-α production has been found in obesity with insulin resistance in both animal models and human subjects [154,322-328] while TNF-α levels decreased after weight lost [322,323]. Moreover, plasma TNF-α levels were reported to be elevated in both NAFLD and NASH patients [251,329] and TNF-α antibody infusions improved hepatic steatosis in ob/ob mice [254]. TNF-α is expressed as a cell surface transmembrane protein and can act in both autocrine and paracrine manners. TNF-α induces lipolysis and inhibits adipogenesis via TNF-R1, the ERK 1/2 pathway, and inhibition of PPAR-γ and lipogenesis [330-332] and it plays a major role in the pathogenesis of insulin resistance in both rodents and humans [150,322,333]. Overexpression of adipose tissue TNF-α mRNA and increased plasma TNF-α levels correlate with increased adipose tissue mass [322,323,334]. At the level of adipose tissue, TNF-α may induce insulin resistance by accelerating peripheral lipolysis with increased release of fatty acids, reducing adiponectin synthesis, and down-regulating the membrane expression of the GLUT4 glucose transporter [45,46,335]. In addition, TNF-α may inhibit lipoprotein lipase activity, reduce the expression of free fatty acid transporters, and decrease the expression of lipogenic enzymes in adipose tissue [323]. TNF-α might induce apoptosis of both preadipocytes and adipocytes.

It was also shown that treatment with insulin sensitizing agents decreased TNF-α concentrations and improved NASH features in both animal models and humans [215,329,336-339].

IL-6

IL-6 is a circulating proinflammatory cytokine that plays a role in insulin resistance [43,304,340-342]. It is primarily secreted by visceral adipocytes and binds to transmembrane receptors to initiate a signal transduction cascade leading to impaired insulin signal transduction via induction of SOCS-3 [343]. Clinical studies have established that plasma IL-6 levels are positively correlated with increased adipose tissue and insulin resistance [333,344,345]. Moreover, plasma and adipose tissue levels of IL-6 are decreased by weight loss [334]. Administration of IL-6 to healthy volunteers induces dose-dependent increases in blood glucose. IL-6 may also increase plasma free fatty acid levels due to its effects on increasing insulin resistance and decreasing adiponectin secretion.

Resistin

Resistin is an adipocytokine first identified in mice that is produced and released by mature adipocytes. In contrast, immune cells rather than adipocytes might be the major producer of resistin in humans [43,346,347]. Its expression is induced during adipocyte differentiation. Its role in insulin resistance is not clear in humans whereas it causes insulin resistance in mice. High resistin levels in the plasma were observed in both genetic (*ob/ob*

and *db/db*) and diet-induced animal models of obesity [348]. Administration of resistin diminished glucose tolerance and insulin action in normal mice and, after the blocking of resistin effects, plasma glucose and insulin levels were decreased in insulin resistant *ob/ob* mice [349]. Whether these finding will be confirmed in humans is not certain.

Regulation of Hepatic Immunity and Increased Sensitivity to Hepatocellular Injury

As regulation of inflammation has become increasing recognized as a central modulator insulin sensitivity, attention has focused on components of innate and cellular immunity [175,350-352]. NKT cells are an important source of proinflammatory cytokines and specific depletion of hepatic NKT cells with consequent proinflammatory cytokine polarization of liver cytokine production exacerbated endotoxin-induced hepatic injury in the leptin deficient *ob/ob* mice [350]. IL-15 administration significantly increased the number of total and liver specific NKT cells, despite persistent leptin deficiency [175]. Additionally, noradrenaline treated *ob/ob* mice showed near normal to normal numbers of hepatic NKT cells and improved the balance between hepatic Th-1 and Th-2 cytokine productions, despite persistent leptin deficiency. These improvements resulted in activation of fibrogenesis in the livers of *ob/ob* mice [175]. Similarly, liver selective NKT cell deficiency and cytokine polarization in the fatty livers of wild-type mice fed with high fat or high sucrose or both had the same effect [353]. In normal biology, NKT cells move to and accumulate in the liver from the thymus. These cells regulate hepatic Th-1 and Th-2 cytokine production (proinflammatory and anti-inflammatory cytokines, respectively) by T cells, NKT cells, and other mononuclear cells in the liver. The selective depletion of hepatic NKT cells might be due to the increased NKT cell apoptosis; induction of fatty liver of dietary induced obese mice promotes hepatic Th-1 cytokine polarization and increased production of both TNF-α and INF-γ, the latter also being increased in the serum [353]. Proposed mechanisms for specific NKT depletion in the liver are decreased rates of NKT recruitment to the liver, decreased hepatic development of NKT cells, increased loss of NKT, or emigration from the liver, and surface markers loss identifying cells as NKT, or any combination of these effects [354]. After endotoxin treatment, inflammation, necrosis, and the concentration of serum liver enzymes as liver inflammation markers were increased significantly [353].

Hepatocyte Apoptosis in NAFLD

Apoptosis, or programmed cell death, is a reflection of normal cell turnover [238]. In the liver, turnover is normally slow and apoptotic cells are relatively rare. Hepatocyte apoptosis was observed more frequently in NASH patients compared to subjects with steatosis alone or control [355]. Fas, which is a death receptor, a surface glycoprotein and a member of TNF receptor family, and caspase activation are two common mediators of hepatocyte apoptosis [238,355-357]. Increased caspase activation and strongly upregulated Fas expression were noted in patients with NASH [355]. Additionally, a positive relationship between the abundance of hepatocyte apoptosis, demonstrated by TUNEL-positive cells histologically, and both the grade and stage of NASH was found, suggesting that apoptosis is not entirely

silent with respect to inflammation, fibrogenesis, and even in the development of cirrhosis [238,355,357]. Oxidative stress is a contributor to hepatocyte apoptosis and ROS increase TNF-α and Fas ligand expression on hepatocytes [234,357,358]. Oxidative stress degrades IκB which is the inhibitor of NF-κB. Activated NF-κB has the capability to induce or inhibit apoptotic events in the hepatocytes (see above). Indeed, NF-κB is a regulator of inflammatory cytokine expression, Bcl-2 family and caspase functions. Hepatic NF-κB expression is increased in patients with NASH [357]. Also, increased Fas expression on the surface of lipid laden mouse (fed a high caloric diet) hepatocytes has been shown [356]. In addition to increased TNF-α secretion, expression of TNF receptor 1 (TNF-R1), a death receptor, was upregulated in patients with NASH [355]. It was recently reported that hepatocyte injury and death in patients with NASH is also associated with increased TNF-R1 mediated apoptosis [238]

It may be that hepatocytes in patients with NASH are more sensitized to death ligands (Fas and TNF-α) due to increased death receptor (Fas and TNF-R1) expression on the surface of these hepatocytes. This could promote apoptosis of hepatocytes via extrinsic stimuli in NASH (death receptor pathway or extrinsic pathway). These events eventually cause cytochrome c release from mitochondria, activation of caspases, mitochondrial dysfunction and other apoptotic events (see above).

Fatty acids-induced hepatocyte apoptosis is discussed previously (see above; free fatty acid toxicity).

HEPATIC FIBROGENESIS IN NASH

Role of Stellate Cells and Cytokines in Hepatic Fibrogenesis

HSC are the main collagen producing cells in the liver and are responsible for fibrosis [359-362]. After activation, HSC proliferate and transform into myofibroblast like cells that lose their retinoid droplets and express α-smooth muscle actin (αSMA). Activated HSC express myogenic markers such as c-myb and myocyte enhancer factor-2, exhibit proinflammatory and profibrogenic properties, migrate and secrete extracellular matrix components (ECM) such as collagen, and regulate the degradation of ECM. Activation of HSC is the crucial step in liver fibrogenesis in a process regulated by autocrine and paracrine factors.

A study of NAFLD patients (16 patients with steatosis alone and 60 patients with NASH) demonstrated that activation of HSC was positive in almost all cases and markedly in two thirds of patients and it was correlated with the degree and location of hepatic fibrosis [359]. Interestingly, this study showed no relationship between the activation of HSC and the severity of necroinflammation and steatosis or stainable iron, but in general, both fibrosis and activated HSC were commonly observed in zone 3 which is also the most affected zone in NASH. HSC activation and upregulation of profibrogenic genes (e.g., collagen α1, and TIMP-1 and -2) were also observed in rats on a high-fat, MCD diet [363]. Additionally, lipid peroxidation associated inflammation and HSC activation with increased TGFβ1 mRNA expression in MCD steatohepatitis models were reported [186,363].

Genetic and environmental factors may affect the development of liver fibrosis in NAFLD. While the genetic factors remain to be elucidated, age, severity of obesity, presence of diabetes, and hyperglycemia are the major non-genetic factors. Elevated plasma glucose, free fatty acids and adipocytokines, which are the important players of NAFLD pathogenesis, activate both Kupffer cells and HSC and eventually stimulate fibrogenesis. Paradis and colleagues investigated the relationship between metabolic factors (hyperglycemia and insulin resistance) and connective tissue growth factor (CTGF), a cytokine that plays a role in the development of liver fibrogenesis, both in vivo in both human NASH and diabetic and obese rats, and in vitro on HSC [364]. In these studies, hepatic CTGF mRNA was overexpressed in all NASH subjects while hepatic CTGF mRNA and its protein were upregulated in *fa/fa* rats (obese and diabetic) compared with their lean littermates. The same group also demonstrated upregulation of both CTGF mRNA and its protein in HSC after exposure to high concentrations of either glucose or insulin. These results correlate with clinical NASH studies and with the pathogenesis of NAFLD. A study demonstrated that insulin resistance is independently associated with the degree of fibrosis in patients with NASH [26] and another study of overweight patients reported that hyperglycemia is a negative prognostic factor in the evolution of NASH towards fibrosis [365]. These effects of glucose and insulin appeared to be independent of TGF-β.

Oxidative stress may also participate in the activation of HSC and the development of fibrosis in NAFLD [186,363,366-368]. The intracellular NADPH oxidase pathway produces ROS and the disruption of NADPH oxidase protected mice from developing severe liver injury. Lipid peroxidation products and leptin also enhance the production of both TGF-β and collagen.

The role of leptin in fibrogenesis remains to be determined despite many efforts to date [168,175,191,293,369]. Initial studies, performed with *ob/ob*, genetically leptin deficient mice, showed that leptin critically regulates liver fibrogenesis [274,277,370,371]. The most probable mechanism for leptin effects is activation of the PI3-K pathway [274]. A direct effect of leptin on HSC in culture has also been reported [372]. Administration of leptin stimulated HSC to upregulate α2 (I) collagen gene expression. Leptin interferes with the production of cytokines (Th-2) such as IL-10 [175] and the balance between proinflammatory Th-1 and profibrogenic Th-2 cytokines regulates fibrogenesis in the liver. Administration of leptin improved Th-2 cytokines and the fibrogenic response of liver in leptin deficient mice. This is an example of an indirect leptin effect on fibrogenesis. The same group also pointed out the relation between NKT cells, which regulate the production of liver cytokines, and leptin. Leptin administration increased the viability and reduced the increased apoptosis rates of NKT cells in leptin deficient *ob/ob* mice. Additionally, the same group showed that norepinephrine, which is a leptin inducible factor, promotes liver fibrosis (see above). A recently performed study of human NAFLD and leptin reported that increased leptin levels in NASH patients simply reflect both increased age and insulin concentrations in the plasma and are not related with the advanced stages of NASH [280].

Angiotensin II, a vasoactive cytokine, plays an important role in liver fibrogenesis [362,373]. Angiotensin II expression is upregulated in the chronically injured liver and induces both hepatic inflammation and fibrogenic actions. It was also shown that decreased renin-angiotensin system activation markedly improved experimentally developed liver

fibrosis. An angiotensin II receptor antagonist, losartan, has been used in hypertensive patients with NASH for 48 weeks and it decreased both plasma TGF-β1 and aminotransferase levels [374]. Additionally, the grade of hepatic necroinflammation, stage of fibrosis, and the amount of iron deposition in the liver were decreased in some subjects.

Hepatocellular Carcinoma

HCC is a late complication in the course of NAFLD that has progressed to cirrhosis [375-381]. Because epidemiologic data attributes the majority of cases of cryptogenic cirrhosis to prior NASH, the hepatocellular carcinoma found to occur in cryptogenic cirrhosis is now also associated with NASH as a predisposing risk [9,14,15,216,382,383]. For unexplained reasons, the characteristic histopathological features of NASH often disappear as the disease progresses to cirrhosis, resulting in an absence of diagnostic criteria in many patients with cryptogenic cirrhosis. The reported the incidence of NASH-associated HCC has been variably reported as 1.73% [9,216], 6.9% [15], 7.31% [378], 13% [382], and 27% [14] among the NASH patients with or without cirrhosis, with or without obesity. Diabetes increases the incidence of HCC by 1.3-2.4 -fold while viral hepatitis causes 13-19 fold increase in the risk of HCC [380]. Additionally, patients with NASH-associated HCC may be slightly older than patients with HCC due to other causes such as alcohol or viral hepatitis [14,15,380,384].

As opposed to human NASH-associated HCC, animal models of HCC can occur in non-cirrhotic livers [60]. It was also reported that increased TNF-α activity might be a necessary component for HCC development besides insulin resistance and fatty liver. Pten is a tumor suppresser gene which is decreased or is absent in some of the primary hepatoma patients. Investigators reported that hepatocytes of mice with hepatocyte specific Pten null mutation showed adipogenic-like transformation, and activated genes of both lipogenesis and fatty acid β-oxidation. The livers of these mice showed a similar histology to human NAFLD and NASH, and then progressed to liver cell adenoma and HCC over time [385]. However, in contrast to human NASH pathogenesis, insulin sensitivity of these mice was increased. Investigators concluded that Pten/PI3K pathways might be involved in the pathogenesis of the development of NASH-associated HCC [385]. An animal model study with hereditary fatty liver showed high incidence of spontaneous development of HCC in non-obese Shionogi mice after one year [386]. Male mice were affected more frequently and earlier than female mice in this study. These mice exhibited progression of disease from fatty liver to NASH, NASH-associated cirrhosis and eventually HCC. However, fld and jvs mice with hereditary fatty liver did not progress to HCC. Similarly, aromatase deficient mice did not develop HCC despite the severe fatty liver [387].

Currently, proposed mechanisms for the transformation from NASH to NASH-associated HCC are severe and cumulative oxidative stress to the hepatocytes, production of damaged DNA, defective or inhibited DNA repair systems, chronic continued hepatocyte injury and inflammatory infiltration, impaired antioxidant systems, and increased cell cycle of hepatocytes. Animal and human studies have also indicated that a connection between age, gender and the disease might be possible.

PATHOPHYSIOLOGY OF THE PATHOLOGICAL FEATURES OF NASH

NAFLD is a clinicopathologic diagnosis. We should bear in mind that the pathogenesis of NASH is accompanied with the histological changes of NASH (Table 3). As mentioned earlier, genetic tendencies and environmental factors cause obesity and insulin resistance. In this background, different mechanisms such as insulin resistance and hyperinsulinemia, increased free fatty acids in the circulation and their toxicity, disturbed production of adipocytokines, increased oxidative stress, iron overload, and mitochondrial dysfunctions induce the development of NAFLD and NASH. Hepatic steatosis is the most frequent and initially observed morphological feature of these processes. Steatosis, inflammation, glycogen nuclei, lipogranulomas, ballooning of hepatocytes, Mallory bodies, and fibrosis are the major features of NAFLD.

Table 3. Histopathologic abnormalities in NASH.

• Steatosis
• Mixed lobular inflammation
• Hepatocyte ballooning with or without Mallory's hyaline
• Variable perisinusoidal fibrosis

Microvesicular and Macrovesicular Steatosis

Increased accumulation of triglycerides as fat droplets within the cytoplasm of hepatocytes is the first step in the development of steatosis. Although two different types of lipid vacuoles as microvesicular and macrovesicular have been identified depending on the size of vacuoles (< 1 micron or vacuoles smaller than the hepatocyte nucleus and > 1 micron in diameter, respectively), the most frequent type found in NAFLD is macrovesicular [388-393]. Mixed type lipid vacuoles are reported as well. Macrovesicular steatosis is typically characterized by a single fat droplet within the cytoplasm of the hepatocyte causing the displacement of the nucleus. In contrast, small lipid droplets and a centrally located nucleus characterize microvesicular steatosis. The observation of microvesicular fat alone is often indicative of causes other than typical NAFLD, particularly rapidly progressive diseases such as acute fatty liver of pregnancy and Reye's syndrome [394].

There may be differences in the causative factors or the development mechanisms between these two types of steatosis. Compared to macrovesicular steatosis, microvesicular steatosis is frequently reported as a consequence of severe mitochondial injury or dysfunction [392,395,396]. This kind of pathology may be genetic such as MTP deficiency, or acquired due to toxins or drugs such as valproic acid and high doses of tetracycline. One possibility is that mitochondrial injury and dysfunction are not so severe in patients with NAFLD as to stimulate the development of microvesicular steatosis. However, as we mentioned earlier, the presence of mixed macro- and micro- steatosis in some NAFLD biopsies is not unusual. An

explanation for this observation might be that mitochondrial injury and dysfunction is substantial enough to stimulate microvesicular development in addition to macrovesicular development, but not so severe as to stimulate a microvesicular development alone. Another possibility is that microvesicular development might develop in a shorter time than that required for macrovesicular development. This idea is supported by the association of acute toxin exposure in the development of microvesicular steatosis. However, we have no information whether such small lipid vacuoles reflect newly synthesized fat droplets, or if the aggregation of micro lipid vacuoles produces macro sized lipid vacuoles over time.

Inflammation

Proinflammatory cytokines, oxidative stress and lipid peroxidation products appear to promote inflammatory infiltration in NASH [21,184, 187,191,397,398]. However, it remains unestablished whether inflammation is primary due to increased proinflammatory cytokines or secondary to the oxidative stress or both. Mixed lobular inflammation, which includes small numbers of polymorphonuclear leukocytes, lymphocytes, and macrophages, is a typical finding in NASH [392,396]. This type of inflammation is usually mild. In contrast, portal inflammation is usually not predominant in adult NASH patients whereas it can be seen in children [399].

Glycogen Nuclei

Glycogen nuclei, or glycogenated hepatocyte nuclei, are complex carbohydrate deposits of the hepatocyte nuclei found in a variety of disorders including diabetes, Wilson's disease and NAFLD [392,400]. They are one of the important pathological changes in diabetics or obese patients. The presence of glycogen nuclei is reported to be a reliable marker for distinguishing diabetics from non-diabetics. Although these are not specific findings or reliable markers for the etiology of NASH, they are commonly seen in diabetic NASH patients (up to 100%) [392,401].

Lipogranulomas

Lipogranulomas are common, seen in up to 82% of patients, but are not specific histologic findings of NASH patients [392,396]. Phagocytic consumption of lipid laden hepatocytes is the main reason of lipogranuloma development. As a consequence, small fat cysts can develop which promote inflammation and eventually lipogranuloma formation. A well-established lipogranuloma contains a central fat vacuole, macrophages, occasional giant cells, and sometimes lymphocytes and eosinophils.

Hepatocellular Ballooning

Ballooned hepatocytes and Mallory bodies are two pathological features described as indicators of ongoing necroinflammation, and are used for grading necroinflammation and as predictors of further stages [402]. At the present time, we have no information whether they are adaptive (physiological), or degenerative (pathological) features of hepatocytes. Only one study carried out in patients with NAFLD has investigated the nature of ballooning hepatocytes to date [162]. This study reported the similarity between the lipid laden hepatocytes and adipose tissue cells. Additionally, few ballooned hepatocytes which had the evidence of hepatocyte degeneration, apoptosis, and necrosis were reported.

Mallory Bodies and Stress Proteins

Stress proteins such as protein p62, HSP 27, and HSP 70 bind other abnormal proteins and form intermediate misfolded proteins [403-405]. Under normal conditions, the ubiquitin-proteasome pathway eliminates these harmful products. When this protective system fails, abnormal cytokeratins accumulate along with p62, HSP 27, HSP 70, ubiquitinated proteins and ropy structures recognized as Mallory bodies develop within ballooned hepatocytes. There are two possible ways for this pathway to fail: production rate of these misfolded proteins that exceeds the capacity of protective systems or inhibition of the protective pathways. The mechanisms of Mallory body formation in humans have not been fully understood yet. Misfolded proteins such as HSPs and other abnormal proteins are the response of hepatocytes to stressors and appear to be degenerative rather than adaptive.

Genetic Susceptibility to NASH and the Basis of NASH-Pathophysiology

In addition to environmental factors, some evidence discussed previously pointed out genetic susceptibility to both development and progression of NASH. For example, although the majority of patients with insulin resistance or metabolic syndrome develop steatosis alone (NAFLD), only a minor group of these subjects progress to advanced stages of NASH. The progression rate of fibrosis is also reported to be variable among NASH patients [17,406-408]. Moreover, both obesity and type 2 diabetes mellitus which have well-established risks of inheritance [409] and are closely associated with NASH. NASH-associated cirrhosis and HCC were also more prevalent among the patients with type 2 diabetes mellitus with or without obesity [10,11,15,390]. Additionally, familial forms of NASH related with lipodystrophy have been reported [410]. Lastly, clustering of both cryptogenic cirrhosis and NASH were reported in kindreds of patients with NASH, besides the familial aggregation of insulin resistance in patients with NASH [411].

NASH Prevalence in Different Racial and Ethnic Groups

A few recently performed epidemiologic studies provided important evidence regarding genetic risks for NASH [8,412-415]. Although two well-known major risk factors of NASH,

obesity and type 2 diabetes mellitus, are more prevalent among African Americans than in Caucasians and Hispanics, epidemiologic studies pointed out significant ethnic and racial variations in the prevalence of hepatic steatosis, NASH, and NASH-associated cirrhosis among these different racial and ethnic groups. Caldwell and colleagues evaluated patients with NASH (159 patients) or cyptogenic cirrhosis (206 patients) and demonstrated only one NASH case and only two cryptogenic cirrhosis cases among African Americans [412]. In contrast, the same study showed overrepresentation of both hepatitis C and hepatic sarcoidosis among African Americans. Browning and colleagues evaluated patients with cryptogenic cirrhosis and reported that cryptogenic cirrhosis-associated with obesity and diabetes is more prevalent among Hispanics and Caucasians, but rare among African Americans [413]. Browning and colleagues also evaluated the impact of ethnicity on the prevalence of hepatic steatosis in a separate study performed with a large, multi-ethnic, population-based sample [8]. Similar to the previously performed two studies [412,413], the authors reported the prevalence of hepatic steatosis to be significantly lower in African Americans than in both Hispanics and Caucasians. Weston and colleagues recently performed a cross-sectional study with newly diagnosed patients with chronic liver disease [414]. The authors reported overrepresentation of Hispanics with NAFLD. It appears that particularly Hispanics with NAFLD may progress to both NASH and cirrhosis more frequently than either blacks or whites. Lastly, Solga and colleagues prospectively evaluated 237 morbidly obese patients undergone bariatric surgery and compared hepatic histopathology features of African Americans with the hepatic histopathology of Caucasians [415]. The authors reported that NAFLD is more common and highly severe among Caucasians. In contrast, African Americans are less likely to have severe NAFLD histopathology. Moreover, Solga and colleagues proposed an African American race-related protection from obesity related liver disease. However, this race-related protection does not cover other chronic liver diseases, such as hepatitis C and hepatic sarcoidosis. Xanthakos and colleagues recently evaluated the prevalence of hepatic steatosis in a population-based cohort of young adult females (aged 24 to 27 years) by magnetic resonance imaging [416]. Of the 281 patients, 56% were African Americans and 44% were white. Although African Americans were significantly more obese and had higher mean leptin and insulin levels and waist circumferences than whites, the prevalence of hepatic steatosis was lower in African Americans than whites. The same study also showed that significant hepatic steatosis was not very prevalent in young adult females despite 42% obesity, 34% central obesity, and 41% elevated fasting insulin in this cohort. These results might reflect differences in the genetic susceptibility of different racial and ethnic groups to both development and progression of NASH.

NAFLD and Genes Associated with Lipid and Glucose Metabolism, Oxidant and Anti-Oxidant Systems, and Proinflammatory Cytokines

Insulin resistance, increased oxidant mediators, decreased antioxidants, and increased production of proinflammatory cytokines are the hallmarks of the pathogenesis of NASH. Thus, investigators evaluated the genes involved in lipid and glucose metabolism, oxidant and antioxidant systems, and the regulation of proinflammatory cytokines [167,207,417-420].

Sreekumar and colleagues investigated hepatic gene expression in patients with NASH-associated cirrhosis, with a particular emphasis on genetic evidence of both insulin resistance

and mitochondrial dysfunction, and compared these results with those of healthy subjects and patients with cirrhosis due to hepatitis C or primary biliary cirrhosis [419]. The authors reported sixteen genes which were uniquely and differentially expressed in cirrhotic-NASH patients. Some of the under-expressed genes are important for free fatty acid metabolism (long chain acyl-CoA synthetase and mitochondrial 3-oxoacyl-Co A thiolase) or important for glucose metabolism (glucose-6-phosphatase and alcohol dehydrogenase). Other under-expressed genes are important for maintaining the mitochondrial functions such as copper-zinc superoxide dismutase, aldehyde oxidase and catalase (important for DNA repair and metabolism). Some of the overexpressed genes are involved in the diminished insulin sensitivity. Additionally, upregulated expression of insulin-like growth factor binding protein-1 and down-regulated expression of apoB 100 were reported while expression of superoxide dismutase-1 (SOD-1) which is involved in scavenging of ROS was found to be decreased in NASH patients. These observations also suggest that impaired repair and metabolism of DNA with increased oxidative mediators and decreased antioxidants might be the cause of mitochondrial DNA mutation and deletion in patients with NASH. Decreased synthesis of apoB 100 in NASH patients, reported previously by the same study group, correlated with the down-regulated expression of hepatic apoB 100. The authors also reported over-expression of some inflammation markers such as hepatocyte-derived fibrinogen-related protein 1, complement component C3, and α-1 antitrypsin in cirrhotic-NASH patients. This evidence further suggests the possibility of a genetic predisposition to NASH.

In another study, Younossi and colleagues studied 91 morbidly obese patients with NAFLD undergone bariatric surgery (27 patients had biopsy-proven NASH) and compared these patients with obese controls [420]. The authors demonstrated differential expression of several hepatic genes and proteins. Most importantly, the authors observed overall down-regulation of phase 2 detoxification enzymes which are important components of the cellular defense system against oxidative stress, such as glutathione S-transferase and cytosolic sulfotransferase isoform 1A2 among three groups (steatosis alone, steatosis and non-specific inflammation, and NASH) and in patients with more advanced stages of NASH, respectively. Increased expression of genes associated with the activation of HSC and fibrogenesis was also reported. These findings were correlated with the proposed mechanisms for the pathophysiology of NASH. Several investigators have also pointed out polymorphisms of the gene sequences encoding the TNF-α promoter, MTP, MTTP, SOD-2, CYP2E1, and apoB 100 may play a role in the pathogenesis of NAFLD [124,125,167,417,418,421].

SUMMARY

NAFLD describes a spectrum of liver abnormalities from benign steatosis to NASH which is characterized by chronic and progressive liver pathology. Although the progression rate of NASH is most likely slower than the other types of liver disease, the prevalence of NASH and its consequences such as cirrhosis and HCC are increasing throughout the world. Currently, our understanding regarding NASH is that adipocytes accumulate excess energy as fat droplets and respond with dysregulated production of adipocytokines. Increased free fatty

acids, predominantly due to peripheral lipolysis and proinflammatory cytokines, interfere with insulin signaling mechanisms to cause both local and peripheral insulin resistance. In addition to increased plasma free fatty acids that are taken up by the liver, insulin resistance, elevated plasma insulin, and elevated glucose levels activate de novo fatty acid and triglyceride synthesis but inhibit mitochondrial fatty acid β-oxidation and export of triglycerides from the liver. Hepatocyte injury and inflammation caused by a number of factors that may include mitochondrial dysfunction, ATP depletion, oxidative stress and lipid peroxidation lead to increased cytotoxic and proinflammatory cytokines and hepatocellular injury. Sustained liver injury leads to hepatic fibrosis, cirrhosis and possibly liver cancer over time.

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Chapter II

NONALCOHOLIC FATTY LIVER DISEASE AND NASH: CLINICAL AND HISTOLOGICAL ASPECTS

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ABSTRACT

Paralleling the increasing prevalence of obesity, diabetes mellitus, and the metabolic syndrome in the general population, nonalcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease worldwide. The diagnosis of NAFLD is established based on evidence of fatty infiltration of the liver in the absence of excessive alcohol ingestion. NAFLD is often diagnosed in asymptomatic persons after the detection of raised aminotransferase during routine screening or evidence of steatosis on ultrasonography. The spectrum of liver injury is variable ranging from simple steatosis with benign prognosis, to nonalcoholic steatohepatitis (NASH) and cirrhosis, conferring an increase in liver-related morbidity and mortality. More advanced stages of NAFLD are associated with older age, higher body mass index, diabetes, hypertension, high triglycerides, and/or insulin resistance. No imaging modality can distinguish NASH from simple steatosis. Liver biopsy remains the only reliable means to determine prognosis based on the severity of fibrosis. The system for histological evaluation for NAFLD/NASH has been proposed by several groups based on a constellation of histologic features rather than any individual feature. The different semiquantitative scoring system for NAFLD/NASH has been used in clinical trials and natural history studies of NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) describes a clinicopathologic condition that is characterized by significant lipid deposition in the hepatocytes of the liver parenchyma in patients with no history of excessive alcohol consumption. NAFLD incorporates a wide spectrum of liver damage ranging from simple steatosis to steatosis plus inflammation and features of hepatocellular damage (nonalcoholic steatohepatitis or NASH) to advanced fibrosis and cirrhosis [1]. Prevalence estimates of NAFLD have used a variety of laboratory and imaging assessments and suggest that NAFLD may be the most common form of chronic liver disease in adults in the United States, Australia, Asia, and Europe, paralleling the epidemic of obesity in developed countries [2-6].

Table1. Causes of Fatty Liver Disease.

Cause	Associations	Steatosis type
Primary		
Acquired insulin resistance	Features of the metabolic syndrome: obesity, diabetes mellitus, hyperlipidemia	Macrovesicular
Secondary	Protein-calorie malnutrition, rapid weight loss,	Macrovesicular
Nutritional	starvation, total parenteral nutrition, bariatric surgery	
Drugs	Glucocoticoids, metrotrexate, isoniazid, allopurinol, synthetic estrogen, α-methyldopa	Macrovesicular
	Tamoxifen, valproic acid, tetracycline, aspirin, cocaine, zidovudine, didanosine, fialuridine, hypervitaminosis A	Microvesicular
	Amiodarone, perihexilene	Phospholipidosis
Toxins	*Amanita phalloides*, *Lepiota*,	Macrovesicular
	Bacillus cereus toxin, petrochemicals, phosphorus	Microvesicular
Metabolic/genetic	Lipodystrophy, dysbetalipoproteinemia, Weber-Christian disease, Wolman's disease	Macrovesicular
	Acute fatty liver of pregnancy, Reye's syndrome	Microvesicular
Others	Inflammatory bowel disease, human immunodeficiency virus infection, small-bowel diverticulosis with bacterial overgrowth	Macrovesicular

Original histopathologic descriptions of NAFLD date back to 1958 when the disease was characterized by Westwater and Fainer [7] in a group of obese patients. Further insights into this disease were made by Peters *et al* [8] in 1975 and subsequently by Adler and Schaffner [9] in 1979. In 1980, Ludwig *et al* [9] described a series of patients who lacked a history of significant alcohol intake but in whom the liver histology resembled that of alcoholic liver disease. They first coined the term "nonalcoholic steatohepatitis" for this condition. Other synonyms have been used to described this entity include fatty liver hepatitis, non alcoholic Laënnec's disease, diabetes hepatitis, alcoholic-like liver disease, and nonalcoholic fatty hepatitis. After much debate, the entity of NASH became accepted, but it is only in the last

10 years that NAFLD and NASH have been widely recognized and diagnosed in clinical practice. NAFLD is increasingly recognized as the hepatic manifestation of insulin resistance and the systemic complex known as metabolic syndrome [11-14]. NAFLD must be differentiated from the steatosis with or without hepatitis resulting from secondary causes such as nutritional conditions, drugs, hepatotoxins, gastrointestinal surgery and some metabolic/genetic conditions as shown in table 1. However, clinicians should consider NAFLD/NASH as a primary diagnosis based on its metabolic associations with obesity, insulin resistance and type II diabetes rather than simply as a disease of exclusion. In several epidemiologic studies, "presumed NAFLD" has been used as a presumptive diagnosis by using the results of abnormal liver enzyme levels, and radiographic studies consistent with fatty infiltration in the absence of other common causes of liver injury. In this chapter we focus on primary NAFLD and discuss the current knowledge of clinical and pathological aspects of NAFLD and NASH.

CLINICAL ASPECT OF NAFLD

Epidemiology

In many developed countries, the prevalence of obesity, diabetes mellitus and the metabolic syndrome has reached epidemic proportions. For instances, in the United States the prevalence of obesity increased from 12% in 1991 to 30.6% in 2002, whereas the prevalence of diabetes mellitus increased from 5% in 1991 to 7.9% in 2001 [15-16]. Similarly, using data from the third National Health and Nutrition Examination Survey (NHANES III), it is estimated that 23.7% of the adult population in the United States suffers from the metabolic syndrome [17]. The dreadful increasing prevalence of obesity and diabetes mellitus as well as the metabolic syndrome in the general population explains why NAFLD has become an increasingly common condition affecting a substantial proportion of the general population. However, the true incidence and prevalence of NAFLD in the general population are unknown at this time.

Incidence

Recently, a historical cohort study [18] was conducted as part of routine health care for employees in a Japanese government office. Most of the employees work in sedentary positions or with only mild physical tasks related to government administration. The subjects were free of previous liver injury, alcohol consumption of more than 140 g/wk, hepatitis B or C infection. Insulin resistance-related features were sought yearly for up to 5 years. Elevated aminotransferases in nonalcoholics were used as a surrogate for NAFLD. The incidence of nonalcoholic hypertransaminasemia was 31 per 1,000 person-years. In comparison between different age groups, the cumulative incidence at 60 months was 14.7% (95% CI: 11.0%, 18.8%) in the 20 to 39 age group and 8.1% (95% CI: 4.6%, 14.1%) in the 40 to 59 age group. To our knowledge, there is no report of the incidence of NAFLD in western countries.

Prevalence

The estimates of the prevalence of NAFLD were obtained from studies that evaluated different patient populations using various methodologies. Because the diagnosis of NAFLD requires liver biopsy with its attendant risk, expense, and uncertain benefit to asymptomatic patients, it is not possible to have population-based estimates of NAFLD. Therefore, biochemical and radiographic surrogates have been used to determine the presence of NAFLD. Published studies of NAFLD can be separated into two general categories: selected population studies and general screening population studies as shown in table 2. Prevalence studies of selected patient samples generally have the advantage of histologic diagnoses of NAFLD but are subjected to both selection and ascertainment bias. The general population screening studies provide more representative prevalence rates, but have limitations due to their diagnostic techniques (liver biochemistries and hepatic imaging methods).

Table 2. Prevalence of NAFLD and NASH.

Population study	**Prevalence (%)**	
	NAFLD	**NASH**
Selected population studies		
Liver biopsy [19-26]	15 -84	1.2-49
Postmortem analysis		
Random deaths [28,29]	16-24	2.1-2.4
Hospitalized deaths [31]	24	
-Lean	36	2.7
-Obese	72	18.5
Surgical patients		
Adult living liver donor [32]	20	-
Bariatric surgery [33-38]	56-86	21-39
General population studies		
Liver enzyme screening [4,6,40]	3.1-23	-
-Lean	1	
-Obese	6	
Ultrasound [41-47]	13-22	-
-Lean	16	
-Obese	76	
Magnetic resonance spectroscopy [48]	33.6	-

Selected Population Studies

In patients undergoing liver biopsy, the prevalence has ranged between 15% and 84% for NAFLD and between 1.2% and 49% for NASH [19-26]. This wide range is related to differences in case ascertainment. One study performed biopsies on patients found to have

fatty liver on ultrasound [27], while others performed biopsies only on patients with chronically elevated liver function tests [19,20,22,23].

Analyses of livers from individuals who died randomly from automobile [28] or airplane [29] crashed showed prevalence rates for NAFLD of 24% and 16%, respectively, while the prevalence of NASH was 2.4% and 2.1%. However, all these studies used selected populations and therefore these data do not reflect the true prevalence of either NAFLD or NASH in the general population [30].

The prevalence of fatty liver and NASH have been estimated from autopsy studies. In a postmortem series of 351 apparently nonalcoholic patients, steatosis was found in 36% of lean and 72% of obese persons and steatohepatitis in 2.7% of lean and 18.5% of obese individuals [31]. However, these results may have been influenced by preterminal events that could have led to a fatty liver.

In healthy young adults being evaluated as donors for living-related orthotopic liver transplantation, fatty liver disease was found in 20%, despite normal ALT levels [32]. In morbidly obese patients undergoing bariatric surgery [33-38], NAFLD was present in 56-78% of patients while NASH occurred in 21-39%.

General Population Studies

Liver function tests have been used in general population screening to diagnose presumed NAFLD. Liver enzymes are not considered to be sensitive or specific either for diagnosing NAFLD or evaluating the severity of disease. Liver enzymes may be in the normal range despite significant liver injury, including fibrosis and cirrhosis [39]. Serum alanine aminotransferase (ALT) has been most widely used to screen for NAFLD, although other enzymes such as aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT) have been used in some studies. The NHANES III, a population survey conducted in the United States between 1988 and 1994 included over 12,000 adults from the general US population. The prevalence estimates of presumed NAFLD ranged from 3.1% using ALT alone [6], to 5.4% using ALT and AST [4], to as high as 23% using GGT as well as ALT and AST [40]. These studies also used different cutoff levels for both abnormal liver enzymes and excessive alcohol consumption. For ALT, lower cut-off levels for men (>30U/L) and women (>19U/L) were proposed recently [27]. Applying this cut-off to the HNANES III sample resulted in a prevalence of elevated ALT activity in men of 12.4% and in women of 13.9%, compared with prevalence of 4.8% in men and 1.7% in women using the cut-off level of the reference laboratory (>43U/L).

An ultrasound screening study for fatty liver was conducted in the general Japanese population; the prevalence was 19% among adults [41]. This figure probably somewhat overestimates the prevalence of NAFLD because it included drinkers. In several subsequent ultrasound studies of Japanese workers, the prevalence was similar to that of the general population and ranged from 15% to 22% [42-44]. The Dionysos study in northern Italy [45,46], used ultrasound to identify fatty liver in order to determine the spectrum and prevalence of liver disease in the general population without evidence of liver disease, diabetes, hypertriglyceridemia and known medications. The result showed fatty liver in 16%

of lean nondrinkers and 76% of obese nondrinkers. Interestingly, elevated liver tests were found in 22% of otherwise normal, healthy controls. In a recent ultrasound study of Chinese administrative officers that excluded “regular drinkers” the prevalence of fatty liver was 13% [47]. The ultrasound screening studies of the prevalence of NAFLD have not been performed in the general US population.

Localized proton magnetic resonance spectroscopy (^{1}H MRS) is an alternative, noninvasive method to measures hepatic triglyceride content (HTGC) and diagnose hepatic steatosis but it has been used only in small research studies. Recently, MRS was used to analyze the distribution of HTGC in 2,349 participants from Dallas Heart Study (DHS) [48]. With using the 95th percentile of normal HTGC of 5.56% as a cutoff, the prevalence of hepatic steatosis in Dallas County was estimated to be 33.6%. Thus MRS provides a sensitive method to measure HTGC and, when applied to a large urban US population, revealed a strikingly high prevalence of hepatic steatosis.

Demographics

The entire histologic spectrum of NAFLD has been reported in all age groups, including children [49,50]. However, the prevalence increases with age, from 2.6% among children to 26% among people 40–59 years old [41,51]. These finding are corroborated by elevated ALT activity screening studies among the general United States adult population (NHANES III), which found the highest prevalence among men in the fourth decade and women in the sixth decade, with the lowest prevalence in older age [52].

Earlier studies suggested a female predominance, range from 65% to 83% of patients [9,54-59], but more recent data suggest an equal to slight male predominance [60-62]. In one study of patients with NAFLD in the United States, men were affected in 68% of cases [52]. The reason for this male preponderance was explained by higher waist-hip ratio in men compared with women. However, females may have an increased tendency to progress to more advanced disease [63,64].

The true prevalence of NAFLD among various racial and ethnic groups is also not fully characterized. A retrospective study looking at hepatology registries have found a lower incidence of NAFLD in African Americans (2% of cryptogenic cirrhosis and 0.6% of NASH) compared with their relative representation in the population [65]. Similarly, in another study, the prevalence of NAFLD was lower among African Americans (1.4%) compared with non-Hispanic Caucasians (7%) [66]. In a small series of diabetic patients, the prevalence of NASH was higher in Mexican American women compared with Whites and African Americans [67]. Mexican Americans were also overrepresented in a small series of pediatric NASH patients [68]. However, such racial/ethnic differences, when found in patient series, may represent true variation or may reflect difference in disease recognition or referral bias. Then, general population survey should be done to avoid this bias. Data from the NHANES showed that the risk of abnormal ALT activity was highest among Mexican Americans in comparison to non-Hispanic, Caucasians and Blacks after adjusting for overall obesity, body fat distribution, and demographic and metabolic factors [52]. Recently, a cross-sectional trial of newly diagnosed cases of NAFLD in the Chronic Liver Disease Surveillance Study [69]

was studied to compare the demographic and clinical features of NAFLD in a racially diverse representative U.S. population. Of the 742 persons with newly diagnosed chronic liver disease, 21.4% had definite or probable NAFLD. The majority were nonwhite and included Hispanics (28%), Asians (18%), and African Americans (3%). African Americans with NAFLD were significantly older than other racial/ethnic groups, and in Asians, NAFLD was 3.5 times more common in males than in females. Clinical correlates of NAFLD (obesity, hyperlipidemia, diabetes) were similar among racial and ethnic groups, except that BMI was lower in Asians compared with other groups. These racial and gender variations may reflect differences in genetic susceptibility to visceral adiposity, including hepatic involvement, and may have implications for the evaluation of persons with the metabolic syndrome. Clinicians need to be aware of the variable presentations of NAFLD in different racial and ethnic groups.

Familial Clustering

Both diabetes and obesity, the risk factors for NAFLD, show familial clustering suggesting that genetic factors may have an important role in the genesis of NAFLD [56,60]. One small study showed that out of eight families, 18 family members with NAFLD, including NASH with cirrhosis were discovered [70]. Another study found that 16 out of 90 patients with NASH had a first-degree relative with the disease [71]. In addition, fatty liver disease has been described in rare familial disorders such as abetalipoproteinemia, and lipodystrophies [72,73]. It serves to illustrate that abnormalities in gene expression may play a role in the genesis of NAFLD. While no familial inheritance pattern emerged, this suggests that environmental as well as genetic factors are likely to have a role in this disease.

RISK FACTORS

The strong associations of NAFLD with obesity, various disorders that include insulin resistance and the metabolic syndrome are documented in a growing body of literature. A recent cohort study by Suzuki *et al* [18] clearly showed chronological ordering of development of risk factors of NAFLD and an association with elevation of aminotransferases levels (nonalcoholic hypertransaminasemia). Weight gain preceded high aminotransferases and other insulin resistance-related features, which appeared sequentially in order as low high-density lipoprotein cholesterol, hypertriglyceridemia, hypertransaminasemia, hypertension, and glucose intolerance. These conditions are very common in the United States, Australian, Asian, and European population and are rapidly increasing in prevalence.

Obesity

The adipocyte is now recognized to be an endocrine tissue capable of secreting a number of adipokines and other substances that may induce insulin resistance [74,75], as part of the pathogenesis of NAFLD. Obesity, defined by a body mass index (BMI) > 30 kg/m^2, is clearly associated with NAFLD [76]. However, NAFLD and NASH may develop in non obese patients. The median prevalence rate of obesity in NAFLD patients was 71%, ranging from 57% to 93% [9,14,56-60,64,76,77]. Virtually all children with NAFLD are obese [53,78]. A number of studies [26,37,56] have established obesity as a risk factor for hepatic steatosis and liver fibrosis. Among Japanese population screening surveys, the prevalence of fatty liver on ultrasound was much higher in obese adults compared with non-obese persons among both men and women [41]. Among such severely obese individuals who underwent liver biopsy at the time of bariatric surgery, the prevalence of steatosis ranged from 74% to 97% [33,34,36,37, 79-83], the prevalence of NASH ranged from 25% to 69.5% [33,79]. Cirrhosis was found in as many as 8% [82,36]. In an autopsy series, three quarters of obese persons had steatosis, while the prevalence of NASH was 18% [31]. Based on these findings, NAFLD may occur in as many as three-quarters of obese people and approximately 20% may have NASH.

It now appears that the distribution of body fat may be more important than the total fat mass. NAFLD patients, even in the presence of normal body weight, have increased visceral adiposity [11]. Visceral fat, rather than total fat mass, has been shown to be a predictor of hepatic steatosis [54,84-86] as well as hyperinsulinemia, decreased hepatic insulin extraction and peripheral insulin resistance [87]. Furthermore, lipolysis in visceral adipose tissue is more resistant to insulin [88], thereby providing a source of hepatotoxic fatty acids in hyperinsulinemic states. Decreasing visceral fat has also been shown to decrease hepatic insulin resistance [89,90].

Recently, there is evidence that obesity has a significant long-term clinical impact on liver disease. In a population-based, cohort study of 11,465 United States adults followed for an average of 13 years, the risk of cirrhosis-related death or hospitalization was increased in overweight and obese persons compared with those of normal weight [91]. The relationship was particularly strong among persons who did not consume alcohol (four times the risk in obese compared with normal weight individuals), providing some indirect support for a causal relationship between obesity and clinically significant NAFLD.

Type II Diabetes Mellitus

After obesity, type II diabetes has been the factor most commonly associated with NAFLD and was reported in 10% to 55% of NAFLD patients [10,53-58,60,61]. There are few studies of the prevalence of NAFLD among patients with type II diabetes mellitus. In two radiographic studies of type II diabetes mellitus, fatty liver was seen approximately 25% [67,92]. The prevalence of steatohepatitis in an autopsy study was 12.2% in diabetics compared with 4.7% among non-diabetics [31]. The extent of steatosis was positively associated with the presence of diabetes [82] and correlated with the degree of impaired

glycemic status, independent of degree of obesity and demographics [83]. A number of studies have shown that hepatic fibrosis is more common in obese patients with diabetes, and that diabetes is an independent predictor for cirrhosis and liver related deaths [93].

Hyperlipidemia

Although hyperlipidemia is frequently cited as a risk, it is unclear how many hyperlipidemic patients have NAFLD or NASH. Hypertriglyceridemia has been reported in 20% to 81% of NAFLD patients [10,53-58,60,61]. Hypertriglyceridemia has also been identified as a predictor both of steatosis on ultrasound examination [48] and of more extensive fibrosis at biopsy in patients with NASH [25]. A high density lipoprotein [HDL] cholesterol level <35mg/dL also almost doubled the risk of NAFLD [40].

A recent study evaluated the dietary habit of NASH patients compared with age-, gender- and BMI-match controls. The results showed that the patients with NASH ate diets higher in saturated fats with less polyunsaturated fatty acids, fiber and the antioxidant vitamins C and E. Interestingly; this study also showed that NASH patients had higher postprandial total triglyceride and very low density lipoprotein (VLDL) triglyceride levels when compared with controls. Also, the postprandial apolipoprotein B48 and B100 levels did not rise with elevated triglyceride levels in NASH patients, as they did in the control group, suggesting a possible defect in the generation of apolipoprotein in NASH patients [94].

Metabolic Syndrome

NAFLD is mainly associated with obesity, diabetes, hyperlipemia, and insulin resistance, which are the main features of the recently characterized metabolic syndrome. The borders of the syndrome, previously known as the insulin-resistance syndrome, have long been unsettled. Recently, the Third Report of the National Cholesterol Education Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III [ATPIII]) [95] provided a working definition of the metabolic syndrome in table 3, based on a combination of 5 categorical and discrete risk factors (central obesity, hypertension, hypertriglyceridemia, low levels of high-density lipoprotein [HDL]-cholesterol, and hyperglycemia), derived from the guidelines of the International Societies or the statements of the World Health Organization [96]. They can easily be measured in clinical practice, and are suitable for epidemiologic purposes.

Data from the NHANES III showed that the prevalence of unexplained elevations of ALT level, which may signify the presence of NAFLD in adults with the metabolic syndrome, was 7% and was significantly higher than in those without the metabolic syndrome [97]. A study of 304 consecutive NAFLD patients without overt diabetes by Machesini *et al* showed that the prevalence of the metabolic syndrome increased with increasing BMI, from 18% in normal weight subjects to 67% in obese subjects [14]. The presence of the metabolic syndrome was significant associated with female gender and age after adjustment for BMI. Of the five criteria for metabolic syndrome, only hyperglycemia

and/or diabetes was significantly associated with NASH after correction for age, gender and obesity, but the simultaneous presence of three or more criteria was associated with different histopathological grading, including a higher prevalence and severity of fibrosis as well as of necroinflammatory activity without differences in the degree of fat infiltration. Logistical regression analysis showed that the presence of the metabolic syndrome was associated with high risk of NASH among NAFLD subjects.

Table 3. Diagnostic criteria for the metabolic syndrome by ATP III proposal 2001.

The metabolic syndrome is present if patient possess three or more of the following criteria:

- *High blood pressure:* if patients systolic and/or diastolic blood pressures were ≥130/85 mmHg or patients were receiving blood pressure lowering drugs
- *Hyperglycemia:* fasting plasma glucose ≥6.1 mmol/L (110 mg/dL) or patients were receiving glucose lowering drugs
- *Hypertriglyceridemia:* fasting plasma triglycerides ≥1.69 mmol/L (150 mg/dL)
- *Low HDL-cholesterol:* fasting HDL-cholesterol <1.04 or 1.29 mmol (40 or 50 mg/dL) in males and females, respectively
- *Central obesity:* waist circumference >88 or 102 cm in females and males, respectively. However, the World Health Organization has recognized the disproportionate contribution of obesity to the development of cardiovascular risk factors in Asians and has provisionally lowered the classification of central obesity to ≥80 or ≥90 cm in females and males, respectively.

Iron Overload

The abnormal iron studies in NASH patients do not necessarily correlate with the presence of stainable iron in liver histology, and conversely siderosis can occur without *HFE* mutation. It has been postulated that insulin resistance itself may lead to iron loading, a phenomenon termed “insulin resistance-associated hepatic iron overload” [98]. This form of iron overload has been suggested recently to be up to 10 times more common than genetic haemochromatosis [99]. Mendler *et al* [98] found that patients with normal transferrin saturation, elevated serum ferritin and siderosis on liver biopsy almost always (94%) demonstrated the insulin resistance syndrome, although only 52% showed NAFLD on biopsy. In support of this, treatment of insulin resistance by strict dietary and antidiabetic control was shown to lead to a reduction in serum iron indices as well as hepatic iron stores in some patients [100], although this has not been confirmed [99].

The role of iron as a cofactor has been studied in NAFLD and NASH, but the results are not clear-cut. In two studies [H39,40], the presence of at least one copy of the C282Y allele was associated with increased hepatic iron and with more advanced hepatic fibrosis. George *et al* [62] showed that the effect was caused by increased hepatic iron concentration induced by the gene mutation. However, another study [101] found that although the presence of an HFE mutation was linked to increased fibrosis, there was no statistical association between

the iron concentration or histological iron score and fibrosis. Other groups have been unable to confirm the association of iron and fibrosis in NASH, and most of the studies addressing the role of iron have concluded that increased hepatic iron content shows no significant association with the degree of fibrosis in these patients [56,60,64,98,102,103].

CLINICAL FEATURES

Symptoms

At the time of diagnosis, similarly to other types of chronic hepatitis, the majority of patients (48-100%) are asymptomatic [10,54,57,58,60] However, in a study by Sanyal *et al* [104], they found fatigue in 73% of patients. As with other chronic liver diseases, the degree of fatigue does not correlate with the severity or the histologic stage of the liver disease [60]. Some patients (48%) may also experience right upper quadrant pain or discomfort [104] secondary to fatty infiltration and stretching of Glisson's capsule. This has been reported to be somewhat more common in children with NAFLD [50,105]. A small fraction of patients experience symptoms indicative of more serious liver disease and may develop pruritus, anorexia, and nausea. The development of jaundice, ascites, variceal hemorrhage, or symptoms of hepatic encephalopathy occurs late in advanced liver disease.

Frequently, the disease is incidentally discovered during routine laboratory examination or work-up of features of the metabolic syndrome such as diabetes, hypertension or dyslipidemia when a hepatic panel is ordered to monitor patients treated with antihyperlipidemic drugs. In another subset of patients, fatty liver is detected when a liver imaging study is ordered for unrelated reason such as workup of suspected gallstone disease.

Signs

There is no pathonomonic sign of NAFLD. The majority of patients are overweight (BMI >25kg/m^2) or obese, and likely to have an elevated waist: hip ratio, indicating abdominal adiposity. The most common finding of liver disease is hepatomegaly, which has been reported in up to 50% of subjects in different studies [10,60]. Clinical stigmata of chronic liver disease are rarely seen on initial presentation. Of the various stigmata known, the presence of spider nevi and palmar erythema are most common [55]. Hypertension is found in 15-68% of cases [10,63,106]. Occasionally, female patients may exhibit increased acne and hirsutism, suggesting the underlying endocrine abnormality of polycystic ovarian syndrome. Acanthosis nigricans, which is hyperpigmented and velvety plaques, most prominent along the flexor lines of the back of neck and axilla, has been reported in 36% to 49% of pediatric patients [78,107]. It is likely to be a cutaneous marker of insulin resistance and frequently identified in patient with excessive weight gain [108].

Laboratory Abnormalities

Mildly to moderately elevated serum levels of aminotransferase are the most common and often the only laboratory abnormalities found in patients with NAFLD. The degree of enzyme elevation is usually between 1 to 4 times the upper limits of reference values. The ratio of AST to ALT is usually less than 1, but this ratio increases as fibrosis advances, leading to a loss of diagnostic accuracy in patients with cirrhotic NAFLD [56]. Serum ALT levels may be completely normal in patients with advanced grade of steatohepatitis or even cirrhosis [39]. It is also known that the degree of ALT elevation does not correlate well with the extent of hepatic damage [109].

Serum alkaline phosphatase may also be variably elevated up to twice the upper limits of normal [10,53,60,61]. Gamma glutamyltransferase levels may be above the normal range in many patients, although their degree of elevation is less than that seen in alcoholic hepatitis [58,110]. Other abnormalities, including hypoalbuminemia, a prolonged prothrombin time and hyperbilirubinemia, may be found in patients with end stage liver disease.

The true sensitivity and specificity of liver enzyme elevations for detection of NAFLD within the general population are unknown. However, the sensitivity and specificity of ALT values have been studied in morbidly obese individuals undergoing bariatric surgery. A cut-off value of ALT level >40U/L diagnosed steatosis with sensitivity of 45% and specificity 100% [79]. While diagnosing steatohepatitis, the sensitivity of the same ALT values remained the same but the specificity decreased to 64%. Using a definition based on an elevated ALT, alkaline phosphatase, or Gamma-glutamyltransferase only modestly increased the sensitivity to 55% and decreased the specificity to 75% for steatosis. For NASH, the sensitivity was 53% and specificity was 50%. Sensitivity in persons not morbidly obese is likely to remain unknown because it is unusual for patients without elevated enzyme activities to undergo biopsy. In one study 81 patients with presumed NAFLD and chronically elevated aminotransferase with other causes of chronic hepatitis excluded with diagnostic serology, underwent biopsy [23]. An elevated aminotransferase level had a positive predictive value of 90% for NAFLD. In another series of 354 patients with abnormal liver enzyme tests in the absence of diagnostic serology, the positive predictive value for NASH was 34% [22].

Hematologic parameters are usually normal unless cirrhosis and portal hypertension lead to hypersplenism.

Ferritin has been reported elevated in 21-62% of patients [62,103,111], but does not usually indicate genetic haemochromatosis, and more likely reflects the hepatic inflammatory process rather than increased iron stored [111,112]. Further, the prevalence of C282Y and H63D mutations has been described as higher [101] or similar [111] to the general population. At the present, testing NAFLD patients for the haemochromatosis gene remains controversial.

In several studies, 10-25% of NAFLD patients have been noted to have a positive antinuclear antibody (ANA), sometimes with a fluctuating pattern [10,113,114]. Furthermore, the overall prevalence of non-specific organ autoantibodies, such as ANA, smooth muscle antibodies (SMA) and anti-mitochondrial-antibodies (AMA) was 35.7% and high titer (>1:100) ANA but not SMA positivity appears to be associated with insulin resistance [115].

A recent study [116] revealed that one quarter of patients with NAFLD had autoantibodies in serum which is significantly higher than the prevalence in the general population. ANA were present in 20% of patients, SMA in 3%, and both antibodies in 2%. Positive autoantibodies were associated with more severe liver histological damage, and higher levels of gammaglobulin. Furthermore, this study showed that 8.9% of presumed NAFLD patients with positive autoantibodies after liver biopsy have fulfilled diagnostic criteria for autoimmune hepatitis (AIH). This study suggested liver biopsy should be done to rule out AIH in most NAFLD patients with positive autoantibodies.

A large body of evidence indicates that NAFLD may stem from a defect of insulin activity. The evaluation of insulin resistance should be part of the diagnostic work-up, unless overt diabetes is present. The euglycemic hyperinsulinemic clamp technique remains the gold standard for the quantitative measurement of insulin sensitivity. However, this method is cumbersome, requires special equipment and is not useful for widespread application. These limitations led to the development of alternative models for assessing insulin sensitivity. The Homeostatic Model Assessment formula, HOMA IR= fasting glucose (mmol/l) x insulin level (μU/mL)/22.5, is a simple way to evaluate insulin resistance [117]. Other methods, such as the quantitative insulin sensitivity check index: QUICKI= 1/ [log ($Insulin_0$) + log ($Glucose_0$)] [118], or a 120-minute oral glucose tolerance test (OGTT) with glucose and insulin determinations, can also be used.

Imaging Studies

The presence of fat in the liver can be diagnosed by using various imaging modalities such as ultrasonography, computerized tomography (CT) scan, and magnetic resonance imaging (MRI). However, none of these modalities can distinguish steatosis from steatohepatitis and are insensitive in detecting steatosis of less than 30% [119].

Ultrasonography is a widely available and low-cost modality. The ultrasonographic findings of diffuse fatty change in the liver are a diffuse hyperechotexture (bright liver) compared with the kidneys, deep attenuation, and vascular blurring as shown in Figure 1 [120]. These parameters allowed diagnosis of fatty liver (defined histologically by fat present in more than 30 % of each lobule) with a sensitivity of 82 to 94% and specificity greater than 82% [92,121-123].

Unenhanced CT remains the optimal technique for imaging hepatic fat; the diagnosis relies on attenuation differences between liver and spleen [124]. Liver fat content also can be semiquantitatively estimated by CT scans [125]. Normally, the attenuation of liver is 50 to 75 Hounsfield units in noncontrast CT scan. With increasing hepatic steatosis, the liver attenuation values decrease by about 1.6 Hounsfield units for every milligram of triglyceride deposited per gram of liver tissue [126]. Thus, in those with a fatty liver, the hepatic attenuation is less than intrahepatic vasculature, giving the appearance of a contrast-enhancement in a noncontrast-enhanced scan as shown in Figure 1 [127,128]. When intravenous contrast is used, the liver attenuation increases but is still lower than the spleen. By CT imaging, the distribution of the fat is unequal with lower attenuation values in the right lobe compared with the left [129]. The sensitivity and specificity of a contrast-enhanced

CT scan are time-and protocol-dependent. Using a cutoff of a liver-spleen differential of 20.5 Hounsfield units 80 to 100 seconds after intravenous contrast injection, a fatty liver could be diagnosed with 86 % sensitivity and 87 % specificity [124]. At 100 to 120 seconds, a difference in hepatic and splenic attenuation of 18.8 Hounsfield units had a sensitivity and specificity of 93 % each [124].

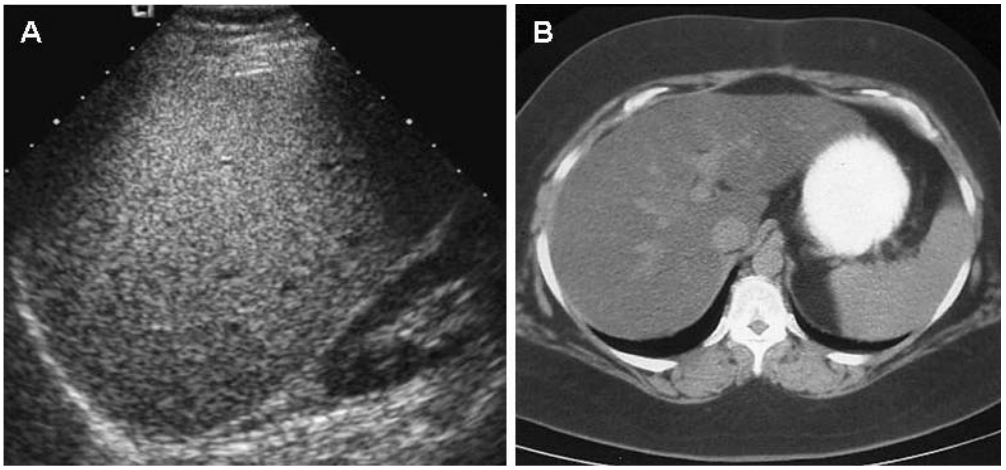

Figure 1. (A) The ultrasonographic findings of diffuse fatty change in the liver are a diffuse hyperechotexture (bright liver) compared with the kidney and vascular blurring. (B) Non-enhanced CT scan through the liver of a patient with fatty infiltration showing low attenuation of the hepatic parenchyma in comparison with the hepatic vasculature giving the appearance of a contrast-enhancement in a noncontrast-enhanced scan.

MRI has a less established role in imaging a fatty liver. The modified spin-echo technique MRI exploits the resonant frequency differences between fat and water. By this method, the fatty liver appears to have a lower signal intensity compared with surrounding muscle [130,131]. MRI for steatosis is more limited in evaluation of patients with iron overload [132]. Localized proton magnetic resonance spectroscopy (^{1}H MRS) is an alternative, noninvasive method to assess hepatic triglyceride content and diagnose hepatic steatosis [40]. Because values given by ^{1}H MRS correlate with liver biopsy results [133-135]. MRS also offers the futuristic prospect of measurement of metabolic parameters, including adenosine triphosphate (ATP) homeostasis in the liver [136,137] and possible lipid peroxidation [138].

In a direct comparison of CT scan with ultrasonography [139], ultrasonography was found to be more sensitive in detecting fatty change. However, CT scan or MRI is superior to ultrasonography when fatty change is focal [140]. In addition, in morbidly obese individuals, the ultrasonographic visualization of the liver may be difficult and poor quality images are obtained.

DIAGNOSIS

The diagnosis of NAFLD can be established only in patients who do not consume significant amounts of alcohol and also requires the exclusion of other liver diseases that may present with steatosis such as viral, autoimmune and metabolic/hereditary liver disease. There is also controversy regarding the precise cutoffs in terms of alcohol consumption in the diagnosis of NAFLD. Confounding this issue is a recent study describing endogenous alcohol production in NASH patients related to the degree of obesity [141], as well as the protective effect of moderate alcohol intake in the prevention of diabetes [33]. In addition, there has been skepticism about the validity of self-reporting as a measure of alcohol consumption. Although there is no consensus regarding the definition of "non-alcoholic" in NAFLD patients, it seems reasonable to exclude patients from this diagnosis if current or within 5 years alcohol intake has exceeded more than 20 g/day in women and 30 g/day in men (12 oz of beer, 5 oz of wine, or 1.5 oz of hard liquor each contain 20 g of alcohol) [142-144].

Several surrogate markers of excessive alcohol consumption over a period of time have been evaluated; there is, however, no perfect test to identify alcohol use, particularly in the context of underlying liver disease. The AST/ALT ratio is usually <1 in patients with NAFLD and may be used to differentiate it from alcoholic liver disease [145]. However, in an ambulatory care setting, alcoholic liver disease has also been found to be associated with a similar AST/ALT ratio [58]. Gamma-glutamyl transpeptidase tends to be higher in alcoholics, at least in hospitalized patients [58]. The mean red cell corpuscular volume is likely to be more discriminative: nearly always elevated in patients with alcoholic liver disease whilst almost never above $98\mu^3$ in patients with NAFLD [26,112]. Other biochemical markers, specifically partially desialylated transferrin (dTf) and the mitochondrial isoenzyme of AST (mAST), have been advocated as tests for active alcohol use in patients with liver disease. In one study [127], the dTf to total Tf (dTf/Tf) ratio of 1.3% or greater was a reliable indicator of excessive chronic alcohol consumption, with a sensitivity of 81% and specificity of 98%. Recently, Stadheim *et al* [146] demonstrated that alcoholic liver disease is not perfectly established by carbohydrate-deficient transferrin (CDT) analysis, although a high total CDT value favors alcoholic liver disease over NASH. Yet, many markers have high accuracy for diagnosing alcohol abuse but low sensitivity for smaller amounts of alcohol [I10].

Histological lesions that have been found to be significantly more common in NASH compared with alcoholic hepatitis are steatosis and periportal glycogenated nuclei [O23], but sclerosing hyaline necrosis, cholestasis and foamy liver degeneration are distinctive histological findings more frequently observed in alcoholic hepatitis than in NASH [O]. These data indicate that distinction between NAFLD and alcoholic liver disease may not always be easy, particularly in those who consume modest amounts of alcohol.

LIVER BIOPSY

The decision of when to perform a liver biopsy in patients with NAFLD sometimes is quite difficult and continues to be an ongoing debate. The aims of liver biopsy for persons suspected to have NAFLD are 1) to confirm the histological diagnosis of fatty liver disease

and exclude other disorders, 2) to distinguish between simple steatosis and steatohepatitis, 3) to determine the risk of progression to more advanced liver disease, and 4) liver biopsy is the best specific means of determining the effect of medical treatment given the uncertain correlation between improvement of liver tests or imaging studies with histologic damage. Recent studies have looked at the utility of performing a liver biopsy in asymptomatic patients with chronically elevated aminotransferase. In a study from the Mayo Clinic prospectively looking at liver biopsies in 36 asymptomatic individuals with elevated transaminase, the presumptive prebiopsy diagnosis was altered in 14% of cases, of which a majority was in those with NASH, and influenced the frequency of subsequent monitoring in 36% [149]. This study was corroborated by a similar study in 81 patients who had no serological evidence of liver disease that showed NAFLD in 51% and NASH in 32% of the biopsy specimens [23]. Recent data suggest that at the time of initial biopsy up to 30-40% of NASH patients will have advanced fibrosis [54,60], and cirrhosis may be found in 10-15% of cases [10,54,55,60]. At the present non-invasive imaging techniques are unable to distinguish steatosis from steatohepatitis, thus a liver biopsy is the only way to establish the diagnosis and stage of NAFLD/NASH. However, some authors suggest that because there is an absence of proven specific pharmacologic treatment for NAFLD, a biopsy is not needed, whereas others believe biopsy provides a sound basis for a conservative approach in many patients with NAFLD. Therefore, the performance of a potentially life threatening procedure requires careful consideration of risk-benefit ratio. Moreover, both the decision to perform a liver biopsy in a patient with suspected NAFLD and the timing of the biopsy must be individualized and should include the patient in the decision making process [13].

Table 4. Predictors of Fibrosis in NASH patients.

Author	N	Mean BMI	Mean age	Predictors of fibrosis
Angulo et al (1999) [56]	144	31.2	50.5	Age $\geq$45 yr, obesity, DM, AST/ALT ratio >1
Marceau et al (1999) [34]	551	47	36	DM, steatosis, age
Garcia-Monzon et al (2000) [79]	46	50.5	41	Obesity, older age, grade of intrahepatic inflammation
Ratziu et al (2000) [26]	93	29.1	49	Age >50, BMI >28 kg/m^2, triglyceride >1.7mmol/L, ALT >2 times of normal value
Dixon et al (2001) [33]	26	47.2	44	Hypertension, ALT >40 U/L, insulin resistance index >5.0
Chitturi et al (2002) [64]	93	32	49	Female, DM, severe liver inflammation
Harrison et al (2002) [63]	102	33.9	51.3	Female, DM, higher AST & AST/ALT ratio

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DM, diabetes mellitus.

Given this debate over whether or not to perform liver biopsies in patients presenting with high clinical suspicion of NAFLD, several studies have established clinical parameter to determine independent predictors of advanced fibrosis to guide the clinician to define groups that may benefit from a liver biopsy. The clinicopathological studies outlined in table 4 demonstrate the independent predictors of fibrosis found in NAFLD patients. Angulo *et al* [56] identified independent predictors of liver fibrosis composed of age >45 years, the presence of obesity or type II diabetes, and an AST/ALT ratio >1. Recent data confirm that patients with NAFLD and type II diabetes develop cirrhosis more often with higher mortality [93]. A clinicobiological score combining BMI, age, ALT and triglycerides (BAAT score) has been proposed to improve obese patient selection for liver biopsy [26]. The BAAT score is calculated as the sum of categorical variables: BMI, age, ALT, and serum triglycerides (each variable score 0-1), ranging from 0 to 4. A score of 0 or 1 would suggest patients without septal fibrosis, thus sparing liver biopsy. Dixon *et al* demonstrated that any two out of the three clinical findings of hypertension, ALT elevation, and a raised insulin resistance index that make up the HAIR index are associated with histological NASH in morbidly obese patients [33]. In this study, portal inflammation and fibrosis were disregarded in diagnosing and staging NASH, but when analyzed separately, they were found to be associated only with hypertension.

HISTOLOGICAL ASPECTS OF NAFLD

The histologic spectrum of NAFLD ranges from pure macrovesicular steatosis to steatohepatitis. Steatohepatitis is a morphological pattern of liver injury, which in nonalcoholic patients may represent a form of chronic liver disease currently known as NASH. The distinctive morphological features of steatohepatitis, regardless of the clinical background, include some “alcoholic hepatitis-like” findings: steatosis, lobular inflammation, which includes polymorphonuclear leukocytes, and perisinusoidal fibrosis in the centrilobular area. Other common features are hepatocellular ballooning, poorly formed Mallory’s hyaline, and glycogenated nuclei [10,53,54,110,150]. NASH can progress to cirrhosis and is increasingly being recognized as a cause for cryptogenic cirrhosis.

Whereas laboratory test abnormalities and radiographic finding may be suggestive of fatty liver, histological evaluation remains the only means of accurately assessing the degree of steatosis, the distinct necroinflammatory lesions and fibrosis of NASH, and distinguishing NASH from “simple” steatosis, or steatosis with inflammation [151].

THE HISTOLOGICAL SPECTRUM OF NAFLD

Steatosis

In NAFLD, the steatosis is macrovesicular droplets that displace the nucleus to the periphery of the cell (Figure 2.); a lesser amount of microvesicular fat may be seen as large numbers of small droplets surrounding a central nucleus. Macrovesicular steatosis results

from complex abnormalities in the delivery, metabolism, synthesis and export of lipids, which result in intracellular triglyceride accumulation. Microvesicular steatosis, considered to be indicative of more severe liver disease, characterizes disease with defective β-oxidation of fatty acids [148]. Then, when the steatosis is entirely microvesicular in type, other etiologies including alcohol and drugs and, where appropriate, acute fatty liver of pregnancy should be considered. An early autopsy study suggested that steatosis in "small" amounts may be present in otherwise normal healthy hepatic parenchyma, and the finding increased with age [28]. The commonly accepted normal value liver steatosis of 5% is based on lipid content measurement [152].

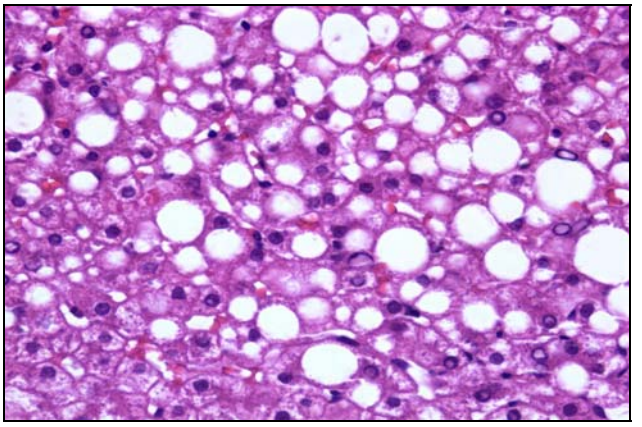

Figure 2. Hepatocytes contain a large vacuole of fat that displace the nucleus to the periphery of the cell.

Steatohepatitis

This is a term that implies the presence of both fatty change and hepatocyte injury accompanied by inflammation. Hepatic injury can be in the form of ballooning degeneration that is reversible, or hepatocyte necrosis or apoptosis that is irreversible.

Hepatocyte ballooning is a structural manifestation of microtubular disruption and severe cell injury [152] and is not unique to alcoholic or nonalcoholic steatohepatitis but is likely a representation of cells undergoing lytic necrosis [153]. Hepatocyte ballooning is characterized by enlargement of the hepatocytes along with rarefaction of the cytoplasm. Ballooned hepatocytes are located most often in centrilobular parenchyma, interspersed with, or adjacent to, regions of steatosis. Hepatocyte ballooning has been identified in studies as a marker for progress in patients with NASH [57].

Apoptotic hepatocytes, seen as shrunken eosinophilic cells with pyknotic nuclei, can be seen in NASH but are never as prominent as in viral hepatitis. Necrotic hepatocytes are not usually prominent, but a mixed inflammatory infiltrate comprising neutrophils, lymphocytes and ceroid-laden Kupffer cells can be seen at the sites where necrotic hepatocytes have disappeared [154].

Mallory's hyaline is defined as a ropy eosinophilic inclusion within hepatocytes that usually is seen in the perinuclear cytoplasm of ballooned hepatocytes located in pericentral parenchyma (Figure 3). It develops as a result of impaired proteosomal degradation of cytoplasmic proteins, predominantly intermediate filaments that bind to ubiquitin [155,156]. The formation of Mallory's hyaline may be the result of defective hepatocellular degradative mechanisms and may play a protective role in the liver [157]. Mallory's hyaline is usually associated with a florid histologic picture of steatohepatitis that includes hepatocyte ballooning, inflammation and pericellular fibrosis [57]. Other causes of Mallory's hyaline are identified without the associated features of NASH such as chronic cholestatic liver disease, copper toxicity, and certain drugs (phospholipidosis associated with amiodarone toxicity), focal nodular hyperplasia, and hepatocellular carcinoma [152,158].

The hallmark of the lobular inflammation in steatohepatitis is the presence of mixed inflammation including small numbers of polymorphonuclear leukocytes within sinusoids and close to ballooned hepatocytes. Mallory's hyaline is chemotactic, and thus affected hepatocytes may be rimmed by neutrophils, this lesion is referred to as "satellitosis". Mild mononuclear cell infiltration may be observed in the lobules or in portal tracts in steatohepatitis in the active or resolving phases [159]. However, when the mononuclear cell infiltration is marked, it may represent concurrent inflammation of another origin such as chronic viral hepatitis C infection. Interestingly, the most common histologic findings of NASH in children are steatosis and lobular mononuclear cell infiltration [70,78,105].

The characteristic pattern of fibrosis that distinguishes steatohepatitis from other forms of chronic liver disease is the initial deposition of collagen in perisinusoidal spaces in the centrilobular and perivenular regions, but it may not be prominent in the earliest stages. In the most prominent cases, individual hepatocytes appear to be outlined by a rim of collagen, giving the liver a chicken wire appearance [148].

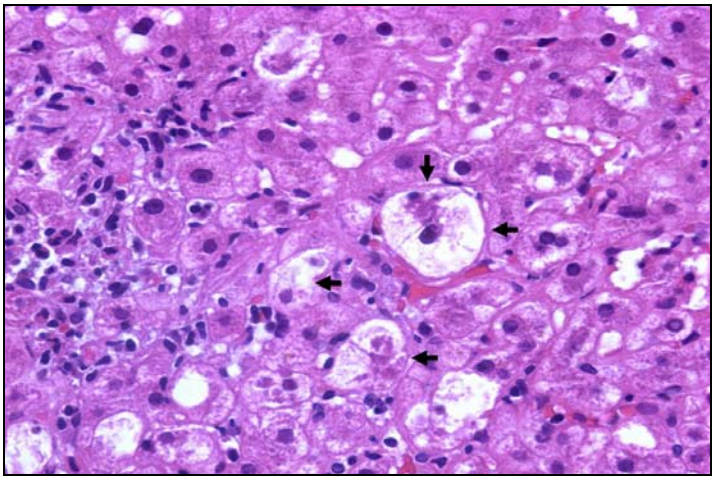

Figure 3. Mallory's hyaline, a ropy eosinophilic inclusion within hepatocytes (↓), usually is seen in the perinuclear cytoplasm of ballooned hepatocytes (←).The lobular inflammation is the presence of leukocytes within sinusoids and close to ballooned hepatocytes.

Other Lesions of Steatohepatitis

Lipogranulomas consist of chronic inflammatory cells, Kupffer cells and occasionally eosinophils surrounding steatotic hepatocytes. They may be localized near terminal hepatic venules, scattered throughout the acinus, or confined to portal tracts [154].

Mitochondrial abnormalities are seen in subjects with NASH by electron-microscopy including megamitochondria, development of multi-lamellar mitochondria, loss of cristae, and presence of intramitochondrial paracrystalline inclusion bodies. Megamitochondria can be recognized by light microscopy as eosinophilic rounded or cigar-shaped intracytoplasmic inclusions in H&E-stained section. Megamitochondria are more commonly associated with chronic alcohol abuse, but may be observed in NASH. Recent studies in NASH indicate that megamitochondria may be more common in periportal hepatocytes and may be indicative of adaptation [160].

The presence of glycogenated nuclei, pseudo-inclusions of glycogen in hepatocyte nuclei, is non-specific, but they are frequently seen in pediatric liver tissue as well as Wilson's disease, diabetes, and NASH [161].

HISTOLOGICAL SCORING SYSTEM FOR NAFLD / NASH

It is now accepted that not all of histologic features of NAFLD are present in each case. Hepatic steatosis, although present in all studies of early stage disease, often decreases and may disappear after the development of cirrhosis [162,163]. This data supports clinical studies relating "cryptogenic cirrhosis" to underlying clinical conditions for NASH [162,163]. Hepatocyte ballooning, Mallory's hyaline, lobular inflammation and pericellular fibrosis also are not present in every patients. This phenotypic variability of the disease has confounded attempts to develop universally accepted criteria for the diagnosis of steatohepatitis.

Matteoni *et al* [57] proposed the term "nonalcoholic fatty liver disease (NAFLD)" to cover a broad spectrum of liver injury, which they divided into four categories. This study showed that cirrhosis developed in 21% to 28% of patients whose index biopsies had shown the combination of lesions of steatosis, inflammation, ballooning, and Mallory's hyaline or fibrosis (NAFLD type 3 and 4), whereas only 4% of patients with simple steatosis (NAFLD type1) and none of the patients with steatosis and inflammation alone (NAFLD type2) had evidence of cirrhosis during the 10 years of follow-up. Until the natural history of subjects with this histologic pattern of NAFLD has been defined prospectively, this will remain a matter of debate.

A system for semiquantitative evaluation of the unique lesions recognized in NASH was proposed by Brunt et al in 1999 [164]. This system was developed to parallel the concepts and terminology used in chronic hepatitis for semiquantitative evaluation, commonly referred to as "grading" and "staging" [165]. The proposed system was based on the concept that the histological diagnosis of NASH rests on a constellation of features rather than any individual feature. The system is summarized in table 5. However, it was developed for NASH and was not developed to encompass the entire spectrum of NAFLD. A different semiquantitative

feature-based scoring system for NAFLD has been developed and used in a recently published treatment trial of this disease [166]. Neither of these systems was designed to evaluate pediatric NAFLD, which may show different histological features than adult NASH [167].

Table 5. Grading and staging of Histopathological Lesions of NAFLD.*

Grading for steatosis
Grade 1: <33% of hepatocytes affected.
Grade 2: 33 to 66% of hepatocytes affected
Grade 3: >66% of hepatocytes affected
Grading for steatohepatitis
Grade 1, Mild:
Steatosis: predominantly macrovesicular, involves<33% or up to 66% of the lobules
Ballooning: occasionally observed; zone 3 hepatocytes
Lobular inflammation: scattered and mild acute (polymorphs) and chronic (mononuclear cells) inflammation
Portal inflammation: none or mild
Grade 2, Moderate:
Steatosis: any degree and usually mixed macrovesicular and microvesicular
Ballooning: present in zone 3
Lobular inflammation: polymorphs may be noted associated with ballooned hepatocytes, pericellular fibrosis; mild chronic inflammation may seen
Portal inflammation: mild to moderate
Grade 3, Severe:
Steatosis: typically > 66% (panacinar): commonly mixed steatosis
Ballooning: predominantly zone 3; marked
Lobular inflammation: scattered acute and chronic inflammation; polymorphs may appear concentrated in zone 3 areas of ballooning and perisinusoidal fibrosis
Portal inflammation: mild to moderate
Staging for fibrosis
Staging (Fibrosis)
Stage 1: zone 3 perivenular perisinusoidal/pericellular fibrosis, focal or extensive
Stage 2: as above plus focal or extensive periportal fibrosis
Stage 3: bridging fibrosis, focal or extensive
Stage 4: cirrhosis

* Modified from Brunt EM [168].

Recently, the Pathology Committee of the NASH Clinical Research Network [169] designed and validated a histological feature scoring system that addresses the full spectrum of lesions of NAFLD and proposed a NAFLD activity score (NAS) with reasonable inter-rater reproducibility that should be useful for studies of both adults and children with any degree of NAFLD. The scoring system comprised 14 histological features, 4 of which were evaluated semi-quantitatively: steatosis (0-3), lobular inflammation (0-2), hepatocellular

ballooning (0-2), and fibrosis (0-4). Another nine features were recorded as present or absent. This system is simple and requires only routine histochemical stains (H&E and Masson trichrome stains). Based on both the agreement data and the multiple regression analysis, the proposed NAS specifically includes only features of active injury that are potentially reversible in the short term. The score is defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2); thus ranging from 0 to 8. Fibrosis, which is both less reversible and generally thought to be a result of disease activity, is not included as a component of the activity score. Cases with NAS of 0 to 2 were largely considered not diagnostic of steatohepatitis; on the other hand, most cases with scores of ≥5 were diagnosed as steatohepatitis. Multiple regression analysis of the scores with respect to the diagnosis of NASH confirmed previous observations that the diagnosis of steatohepatitis is not dependent on a single histological feature, but rather involves assessment of multiple independent features. One concern for any new scoring system is how it applies in actual clinical trials.

SAMPLING VARIABILITY OF LIVER BIOPSY IN NAFLD

The basic assumption in liver biopsy is that the small fragment collected through percutaneous liver biopsy is representative of overall hepatic involvement. However, multiple studies have shown considerable sampling variability for most histologic features including cirrhosis when more than 1 sample is analyzed [170-176]. This sampling variability has the potential to alter significantly the diagnosis and staging of NAFLD. Janiec *et al* reported 10 morbidly obese patients who underwent simultaneous liver biopsies from the right and left hepatic lobes during an open examination preceding Roux-en-Y gastric bypass surgery. Liver biopsy samples taken from the right and left hepatic lobes showed similar grades of disease activity, but differed in histopathologic staging in 30% of the NAFLD patients. Obtaining an adequately sized biopsy (>1.0 cm in length with >10 portal tracts) greatly reduces sampling error [177]. However, in patients with NAFLD, liver biopsy is performed in most cases via an intercostal route for both diagnostic purposes and therapeutic trials. Thus, percutaneous liver biopsy studies may be a better reflection of this issue that can be encountered in clinical practice. Recently, Ratziu *et al* [178] revealed that histologic lesions of 2 liver samples of patients with NASH assessed by percutaneous liver biopsy in the right lobe of liver through the same intercostal route using ultrasound guidance are unevenly distributed throughout the liver parenchyma. Agreement between the 2 biopsy specimens was only moderate for most features, including hepatocyte ballooning and perisinusoidal fibrosis, whereas, for some others, such as acidophilic bodies, lobular inflammation, or Mallory's hyaline, the agreement was poor. Only steatosis grade and interface hepatitis displayed substantial agreement between the 2 biopsies, whereas there was no high agreement observed for any of the histologic features that were under study. Therefore, sampling error of liver biopsy can result in substantial misdiagnosis and staging inaccuracies that might carry significant implications for clinical management in an era when pharmacologic therapies for NASH are slowly emerging.

NATURAL HISTORY OF NAFLD

Despite being common and potentially serious, the natural history of NAFLD remains poorly defined. Based on epidemiology studies, the prevalence of NAFLD in the United States may be as high as 30%. This, together with an accumulating body of evidence that some patients with NAFLD can progress to cirrhosis, liver failure, and hepatocellular cancer (HCC) has emphasized the need for detailed information on the natural history of NAFLD both to guide patient management and to enable rational public health care planning. Natural history studies reported to date can be divided into 2 main categories; 1) serial biopsy studies looking for evidence of histological progression in patients with different stages of NAFLD and 2) cohort studies examining the clinical outcomes of patients with NAFLD diagnosed histologically or ultrasonographically. The principal limitation of the majority of these studies have been their relatively short-term follow-up, and for the serial biopsy studies in particular, a high degree of selection bias in patients undergoing repeat biopsy [179].

Table 6. Fibrosis progression in NAFLD: studies with serial biopsies.

Author	No.	Average F/U (years)	Worsened (%)	No change (%)	Improved (%)	Basal factors associated with fibrosis progression
Lee (1989) [54]	13	3.5	38	62	-	No factors
Powell (1990) [60]	13	4.5	46	46	8	NA
Bacon (1994) [55]	2	5	50	50	-	NA
Ratzui (2002) [26]	14	5	14	57	29	NA
Evans (2002) [180]	7	8.2	57	43	-	NA
Harrison (2003) [181]	22	5.7	32	50	18	Higher serum ALT
Fassio (2004) [182]	22	4.3	32	68	-	Obesity
Adams (2005) [183]	103	3.2	37	34	29	DM, higher BMI, low initial fibrosis stage

NA, not assessed; ALT, alanine aminotransferase; DM, diabetes mellitus; BMI, body mass index.

NAFLD may progress to steatohepatitis and cirrhosis with its complications. It is uncertain what proportion of patients has progressive disease and it remains unclear whether some factors predict higher rates of progression. Fibrosis stage is recognized as the most objective indicator of liver damage and is the best prognostic marker for morbidity and mortality in liver disease of various etiologies. Several studies have investigated the natural history of NAFLD by examining fibrosis stage among patients with paired liver biopsies as shown in table 6. The earlier published results of repeat liver biopsies come from 78 patients with NASH but no cirrhosis (included in seven different studies) [26,54,55,60,180-182]. The second biopsies were performed 1.2 to 15.7 years after the first and showed fibrosis progression in 37.2% of patients [26,54,55,60,180-182]. The first four studies were clinical series examining NASH in which only a minority of patients underwent a repeat biopsy

[26,54,55,60], whereas the last three studies were specially designed to evaluate histological changes [26,180-182]. These studies examining fibrosis change over time have been limited by small numbers. In addition, patients have generally undergone sequential biopsies due to clinical indications, potentially biasing results towards patients with more severe or atypical disease. Recently, Adams *et al* [183] reported 103 patients with NAFLD that in the majority underwent a biopsy at a predetermined interval as part of a clinical protocol, therefore, limiting this type of selection bias. Fibrosis stage apparently progressed in 37%, remained stable in 34% and regressed in 29%. Severity of steatosis, inflammation, hepatocyte ballooning and Mallory's hyaline improved significantly. Aminotransferases decreased significantly between biopsies, paralleling improvement in steatosis and inflammatory features but not fibrosis stage. The rate of fibrosis change ranged from −2.05 to 1.7 stages per year. By multivariate analysis, diabetes and low initial fibrosis stage were associated with higher rate of fibrosis progression, as was higher BMI when cirrhotics were excluded.

From these series, it is estimated that approximately one-third of patients had worsening histology: as many as 20% developed worsening fibrosis and up to 20% progressed to cirrhosis over approximately 5-7 years. Risk factors for progression remain unclear although a number of studies have examined predictors of more advanced fibrosis on the baseline biopsy. However, it should be emphasized that all of the predictive factors in predicting more severe histology on the baseline diagnostic biopsy may be used to predict a higher rate of fibrosis progression on the histological course of NASH unless patient undergoes repeat biopsy.

Table 7. Cohort studies of clinical outcomes of different stages of NAFLD.

Author	Population	No.	Average F/U (years)	Cirrhosis prevalence (%)	Liver-related deaths (%)	Overall deaths (%)
Teli (1995) [61]	Simple steatosis	40	9.6	0	0	35
Dam-Larsen (2004) [184]	Simple steatosis	109	16.7	1	0.9	24.8
Lee (1989) [54]	NASH	39	3.8	16.3	2.6	26
Powell (1990) [55]	NASH	42	4.5	7	2.4	4.8
Evans (2002) [21]	NASH	26	8.7	3.8	0	8.7
Fassio (2004) [182]	NASH	22	4.3	0	0	0
Hui (2003) [185]	NASH-cirrhosis	23	5.0	100	21.7	26
Matteoni (1999) [57]	NAFLD(NASH)	98(73)	8.3	20(25)	9(11)	49(40)
Adams (2005) [186]	NAFLD(NASH)	420(49)	7.6	5(NA)	1.7(8)	12.6(35)

The clinical outcomes of NAFLD based on the initial histologic classification from cohort studies can be summarized (table 7) as follows: contrary to previous dogma, simple steatosis can progress to steatohepatitis, fibrosis, cirrhosis, and even liver-related death but progression occurs in less than 5% of patients over a 8–17 year follow-up with no impact on overall mortality [57,61,184]. The lack of impact on mortality of simple steatosis, initially observed in a Danish study, has recently been confirmed by 10-year follow-up data from the Dionysos Study in Northern Italy [187]. Patients with NASH, with or without fibrosis, can progress to cirrhosis assessed histologically or clinically over a 3–8 year follow-up, with proportions ranging from 0% in the least selected follow-up studies [182] to 25% in the

largest clinical follow-up study performed to date [57], although this study may have included patients with NASH and cirrhosis in the "NASH" group [188]. Why some patients with NAFLD progress to fibrosis and cirrhosis and others generally have a benign course without progressive clinical and histological sequelae is unclear. Based on similar age at presentation and the long-term stability of NAFLD type 1-2 compared to the risk of progression in NAFLD type 3-4, it is likely that these two entities originate separately and probably become different early [57]. Once cirrhosis develops in patients with NAFLD the prognosis appears to be poor with two studies reporting that up to one-third of patients develop liver-related morbidity or mortality over a relatively short follow-up period [185,189] with one reporting a high rate (27%) of HCC [189], consistent with a several other reports of HCC developing in patients with NASH cirrhosis [190]. This high liver-associated death rate in NASH cirrhosis presumably accounts for the 10% liver-related death rate reported in the only NASH follow-up study [57].

Recently, a large cohort study of community-based patients from the Mayo Clinic is the first to describe the natural history of NAFLD [191]. The mean length of follow-up was 7.6 years. Mortality was significantly increased among patients with NAFLD compared with the expected mortality of the general population of same age and sex and was predicted by presence of impaired fasting glucose /diabetes, cirrhosis, and older age. Death occurred in 12.6% of patients and was most commonly due to malignancy and ischemic heart disease, which are also the two most common causes of death in the Minnesota general population. Liver disease was also an important contributor of death among patients with NAFLD, being the third most common cause and accounting for 13% of all deaths (as compared with the 13th leading cause of death among the Minnesota general population, accounting for <1% of all deaths). This implies that the increased overall mortality rate among NAFLD patients compared with the general population is at least partly due to complications of NAFLD. Nevertheless, the incidence of liver-related death was low (1.7%) as was the occurrence of cirrhosis (5%) and cirrhosis-related complications (3.1%). Liver histology was adequate for accurate staging in 61 patients, with 49 fulfilling the histological criteria for NASH, 10 having simple steatosis, and 8 having established cirrhosis. Patients undergoing liver biopsy were more likely to have symptoms, diabetes, and clinical evidence of advanced liver disease and also had a significantly lower survival than those who did not undergo liver biopsy (10-year survival 55% versus 90%). None of the 10 patients with histologically proven, simple, ("bland") steatosis developed clinical evidence of cirrhosis or died from a liver-related cause, confirming the relatively benign natural history of the mildest form of NAFLD demonstrated in previous studies. The 8% liver-related mortality in the 49 patients with histologically proven NASH is similar to the 10% reported in the only equivalent study reported to date [162]. In addition, the outcome in patients either with biopsy-confirmed cirrhosis at entry or developing clinical cirrhosis during follow-up, confirms the poor prognosis of patients with NASH cirrhosis, with 33% dying from a liver-related cause and one patient developing HCC, consistent with the previous studies examining the natural history of NASH-related cirrhosis [180].

Therefore, from both categories, it would appear that the natural history of NAFLD depends critically on disease stage as shown in Figure 4. Patients with simple steatosis have a relatively benign "liver" prognosis with a risk of developing clinical evidence of cirrhosis

over 15–20 years on the order of 1%–2%. Patients with NASH and fibrosis can progress to cirrhosis, defined histologically or clinically, with the risk varying from 0% at 5 years to 12% over 8 years [57,182]. Once cirrhosis develops, patients are at high risk of developing hepatic decompensation and of dying from a liver-related cause including HCC. Despite the high prevalence of obesity, diabetes, and the metabolic syndrome there is, as yet, no evidence that patients with NAFLD have an increased risk of death from either malignancy or cardiovascular disease. The lack of increased death rate from malignancy may simply be due to the still relatively small studies that have not examined cause-specific standardized mortality ratio, while the lack of increased mortality from cardiovascular disease may be attributable to the putative "cardioprotective" effects of chronic liver disease including reduced arterial pressure, an improved lipid profile or prolonged coagulation parameters [192]. Clearly, what is required are larger and longer follow-up studies in patients with histologically defined NAFLD, ideally comprised of both serial biopsies and clinical observations to include a detailed examination of the incidence/prevalence of malignancy and cardiovascular disease. We will then be able to provide patients with accurate prognostic information, initiate treatment trials on a more rational basis and predict the likely burden of NAFLD-related end-stage liver disease on health care systems [179].

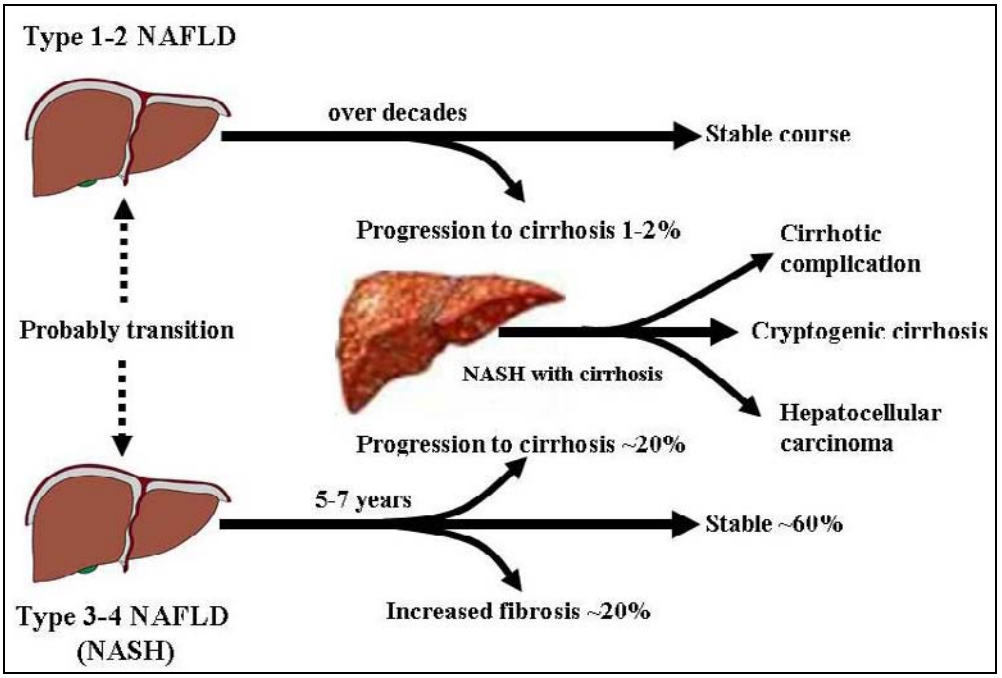

Figure 4. The outcome of NAFLD based on initial histological classification.

SUMMARY

NAFLD describes a clinicopathologic condition that is characterized by significant lipid deposition in the hepatocyte of the liver parenchyma in patients with no history of excessive

alcohol consumption. NAFLD is increasingly recognized as the hepatic manifestation of insulin resistance and the systemic complex known as metabolic syndrome. NASH, the most severe form of NAFLD, is emerging as a common, clinically important type of chronic liver disease in industrialized countries, and rate are increasing in many developing countries. The prevalence rate of NAFLD and NASH are expected to increase worldwide, concurrent with the epidemic of obesity and type II diabetes. They are now estimated to be in the range 3.1-33.6% for NAFLD and 1.2-49% for NASH. The majority of patients with NASH are asymptomatic. When present, clinical features such as fatigue, hepatomegaly and hepatic discomfort are non-specific. Despite recent advances in technology, physicians must still rely on the liver biopsy for diagnosing and particularly for staging liver disease. Recently, the Pathology Committee of the NASH Clinical Research Network designed and validated a histological feature scoring system that addresses the full spectrum of lesions of NAFLD and proposed a NAFLD activity score (NAS) that should be useful for studies of both adults and children with any degree of NAFLD. The long term prognosis for patients with NAFLD appears to depend on the initial histology. NAFLD type 1 and 2 are relatively stable conditions. NAFLD type 3 and 4 (NASH) is potentially progressive, with approximately 20% of patients having increased fibrosis and up to 20% progression to cirrhosis over 5-7 years. Once cirrhosis develops, patients are at high risk of developing hepatic decompensation and of dying from a liver-related cause including HCC.

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Chapter III

THE TREATMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE- AN ENTITY IN EVOLUTION

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a common phenomenon being the hepatic manifestation of the metabolic syndrome. It may be associated with significant morbidity and mortality. At present liver biopsy is required in order to differentiate benign disease from progressive disease. The majority of evidence supports weight loss and lifestyle changes as the major treatment intervention. Other treatments including bariatric surgery, insulin-senstizing agents including metformin and thiazolidinediones,lipid lowering agents, anti-oxidants and ursodeoxycholic acid have alos been investigated. In this review the evidence is reviewed and a proposal for treatment presented.

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases in the United States and Europe (1). It was first noted by Ludwig et al in 1980 to describe a cohort of obese female patients with non-insulin-dependent diabetes in whom the hepatic histology was suggestive of alcoholic hepatitis but there was no history of alcohol abuse (2). It is now clear that there is a strong connection to obesity and insulin resistance. NAFLD is now regarded as the hepatic manifestation of the metabolic syndrome.

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INTRODUCTION

The exact prevalence of NAFLD is uncertain, ranging from 16-23% in liver biopsy studies to 15-39% in ultrasound studies (1). Although there has been a surge of interest in NAFLD in recent years, there are no clear recommendations regarding the most effective treatment.

In general, medical treatment for a specific disease is best given when several criteria are fully addressed:

1. The natural history of the disease is well defined
2. It is possible to reliably identify patients who require treatment
3. The treatment will halt the natural progression of the disease, or cause a regression in the disease and improve the quality and/or the length of the life of the patient
4. The side-effects of the treatment are tolerable in comparison to the morbidity of the illness and the treatment is cost effective.

NAFLD is a recently recognized condition and its prevalence is increasing concomitant to the epidemic of obesity affecting the developed countries. This has resulted in the situation where a common disease that can cause significant morbidity and mortality exists and there is still a lack of reliable data on which to base diagnostic and therapeutic decisions.

The purpose of this review is to examine the available evidence regarding the treatment of NAFLD.

NATURAL HISTORY OF NAFLD

The natural history of the disease is not well defined, partly because of different exclusion criteria for alcohol and partly because of different criteria for diagnosis- such as imaging studies or histological criteria. Although NAFLD was initially believed to be a benign, non-progressive disease it is now clear that a subset of patients can develop cirrhosis, end-stage liver disease and hepatocellular carcinoma. Recent data show that NAFLD is a common cause of cryptogenic cirrhosis [1]. However, more than 40% of an octogenarian population were found to have ultrasound evidence of NAFLD, implying that its presence does not necessarily impact on longevity [3]. Thus NAFLD is a common disease that can result in cirrhosis in some, but not all patients, and whose natural history is unclear.

IDENTIFICATION OF PATIENTS WITH NAFLD WHO REQUIRE TREATMENT

It is thought that patients with NASH are at risk for progression to fibrosis and cirrhosis, whereas patients with steatosis alone tend to have a relatively benign course [4]. Although NAFLD may be identified by imaging techniques such as ultrasound or CT scan, there is no

accurate or reliable method of identifying the patients with steatohepatitis or fibrosis from those with just steatosis, except for liver biopsy [1]. In addition it appears that there may be marked variability in the pathology within the liver, hampering interpretation of studies performed on single biopsies [5]. There are no consensus recommendations available but many people would perform a biopsy if there is marked ALT elevation (> x 2 above the upper limit of normal), AST>ALT, or failure of liver enzymes to decrease after initial lifestyle and dietary modifications [1].

Thus one of the problems in trying to assess the utility of treatment for NAFLD is assessing the pre-treatment severity of disease.

Weight Loss and Non-Pharmacological Treatments

There is a strong association between the metabolic syndrome and NAFLD. Since lifestyle changes including weight loss and increased physical activity have a positive effect on many of the parameters of the metabolic syndrome, it is reasonable to expect a favorable effect of a similar program on NAFLD. For example, in a group of 3234 nondiabetic patients with impaired glucose tolerance, a lifestyle modification program consisting of a mean of 7% decrease in weight and 150 minutes of physical activity per week resulted in a reduction in the development of diabetes of 58% [6]. It has recently been shown that in obese patients, weight loss of as little as 8 kg can lead to reduced fat content in the liver (assessed by ^{1}H magnetic resonance spectroscopy) , improve insulin sensitivity and return fasting blood glucose to normal [7]. There are a total of 6 studies in the literature regarding lifestyle changes and the effect on NAFLD [8-13]. These studies are summarized in Table 1.

Table 1. Peer reviewed published trials of lifestyle changes in NAFLD.

Name	Type	Evidence level	Treatment	Control	Number	Time (m)	Biopsy
Andersen	Case series	2b	Diet	x	41	4-23	variable
Vajro	Case series	2b	Diet, exercise	x	9	30	Improved
Ueno	Open label	2b	Diet, exercise	No Rx	25	3	Improved
Franzese	Case series	2b	Diet, exercise	x	42	6	nd
Hickman	Case series	2b	Diet, exercise	x	10	15	Improved
Huang	Case series	2b	Diet	x	16	12	ns

Andersen et al [8] reported a case series of 41 obese patients with NAFLD entering a weight-reducing program. A median weight loss of 34 kg in a 4-23 month period was

achieved resulting in a significant decrease in the amount of fatty infiltration of the liver and in a significant improvement of liver enzyme tests.

Vajro et al [9] reported the results of a study including nine obese children with chronic (up to 49 months) elevation of serum transaminases. Following a hypocaloric diet there was a decrease in the serum transaminases and in the brightness of the liver on ultrasound. Ueno et al [10] treated 15 obese NAFLD patients with a program of restricted diet (25 kcal/kg of ideal body weight) and exercise for 3 months. The exercise regimen was intense, consisting of 3000 steps of walking per day which was increased by 500 steps every 4^{th} day up to 10,000 steps followed by jogging twenty minutes twice per day. This resulted in an average decrease of BMI of 3 kg/m^2 and a decrease in serum aminotransferases, cholesterol and glucose. On repeat liver biopsy a decrease in steatosis was found although there was no change in the necroinflammatory score. There was no clinical or histological change in a control group of 10 patients who were not on the program. However, this regimen is probably unlikely to be achieved and maintained in most populations. Franzese et al [11] reported 42 obese Italian children with either elevated liver enzymes or an ultrasonographic picture of fatty liver who were evaluated by serial examination of serum enzyme levels and ultrasonography of the liver one, three and six months after starting a hypocaloric diet. All patients who lost at least 10% of their ideal body weight in the 3-6 months follow up had either normalization or improvement of the ultrasonographic findings.

Even moderate weight loss may have a beneficial effect. Hickman et al have reported their experience with 27 patients with hepatitis C virus (HCV) infection and hepatic steatosis and 16 patients with non-HCV associated hepatic steatosis (10 out of 16 of these patients had clinical and histological diagnosis of NAFLD). The patients had an initial 3 month period of weight reduction followed by a 12 month program of weight maintenance and 150 minutes of aerobic exercise per week. The mean weight loss was 4%. There was a decrease in ALT levels and fasting insulin levels in those who lost weight and maintained the weight loss. In addition there was an improvement in the health related quality of life. However, since the results were presented for the whole group, it is not possible to ascertain the exact effect of the intervention in the unequivocally NAFLD group.

Recently, the results of a study on 16 patients with biopsy-proven NASH who completed 12 months of dietary intervention and in whom 15 had repeat biopsies was reported [13]. The diet chosen was based on 40-45% of daily calories from carbohydrates, 35-40% from fat especially mono and polyunsaturated fats and 15-20% protein. This intervention resulted in a non-significant decrease in weight, waist circumference, visceral fat, fasting glucose, insulin resistance, triglycerides, AST, ALT and histological score (modified Brunt system). Interestingly, the nine patients who had a histological response to therapy had a significantly greater reduction in weight and waist circumference.

Another recent study reported at Digestive Disease Week of 5 patients with NASH on liver biopsy who were given a very low carbohydrate diet (25 g/day) for 6 months. At the end of the study there was an improvement in ALT levels and hepatic steatosis and histological grade [15], although it was not possible to distinguish between the effects of a low carbohydrate diet and generalized weight loss.

Bariatric Surgery

Rapid weight loss after bariatric surgery has been associated with transient worsening of inflammation and fibrosis.

Weight loss is notoriously difficult to achieve and maintain and recently bariatric surgery has been gaining acceptance as a treatment for morbid obesity. Many of these patients have NAFLD as well.

Ranlov et al [16] reported on 15 patients who were reexamined 1 year after bariatric surgery -gastric bypass (7 patients) or gastroplasty (8 patients). The incidence of steatosis had decreased from 73% to 40% but there was no fibrosis present in the biopsy samples.

Luyckx et al [17] treated 528 patients with gastroplasty, of whom 69 with a marked weight loss were evaluated before and after a mean of 27 ± 15 months including repeat liver biopsy. Forty-five percents of the biopsies were considered as normal (vs 13% before, $P < 0.001$) while pure steatosis was still observed in 38% of the patients (vs 83% before, $P = 0.001$). Although the severity of the steatosis was significantly reduced there was an increase of hepatitis (26% vs 14% before, $P < 0.05$)

Dixon et al [18] reported their experience of 36 patients who underwent laparoscopic adjustable gastric band placement. These patients had paired liver biopsies, at the time of laparoscopic placement of the adjustable gastric band and the second after weight loss, at a mean of 25.6 ± 10 months after band placement. The mean weight loss was 34.0 ± 17 kg. The second biopsy demonstrated improvement of lobular steatosis, necroinflammatory changes and fibrosis, although portal abnormalities remained unchanged (Figure 2). There were 23 patients who had the metabolic syndrome before surgery and they tended to have more extensive changes before surgery and greater improvement after weight loss.

Kral et al [19] reported on 689 obese patients who underwent biliopancreatic diversion of whom 104 underwent routine biopsy at reoperation. Severe fibrosis (grade 3-5) decreased in 28 patients but mild fibrosis (grade 1-2) appeared in 42 patients. Overall fibrosis and inflammation decreased over time ($P<.01$). The 11 patients who had cirrhosis exhibited decreased fibrosis from a mean grade 5 to grade 3, as well as reduced inflammation, Mallory bodies, and glycogenated nuclei. Seven patients had disappearance and 2 regression of nodules and fibrous bridging. Despite these favorable results, too rapid weight loss may be deleterious. There are reports of hepatic decompensation in some patients with NAFLD and exacerbation of steatohepatitis in others following bariatric surgery [20-22]. This is thought to be due to massive fatty acid mobilization from visceral stores, reaching the liver through the portal vein.

Andersson Friis-Liby and colleagues [23] studied the early changes in liver tests and in intrahepatic fat (by computed tomography) during rapid weight loss (overall weight loss was about 28 kg) in 40 patients with NAFLD. An initial increase of fatty infiltration in the liver was seen, in parallel to an increase in ALT levels. Thereafter, weight reduction induced normalization of liver fat and improved serum ALT and insulin sensitivity.

Recently, three reports were presented at the Digestive Disease Week meeting. Kaushik and colleagues [24] assessed the effects of Roux-en-Y gastric bypass surgery on liver histology in 31 obese patients with NAFLD. Mean BMI decreased from 51 kg/m^2 to 34 kg/m^2. All patients had steatosis on initial biopsy, but only 39% had steatosis on follow-up;

68% of subjects showed improvement in NASH grades and 23% had no inflammation following Roux-en-Y gastric bypass.

Barker and colleagues [25] also reported that weight loss, achieved through Roux-en-Y gastric bypass, improved histopathology in 149 obese patients with biopsy-proven NASH. At the time of surgery, 23% of patients had histopathologic evidence of NASH. After an average of 642 days, histopathologic criteria for NASH were no longer found in 84% of patients. Surgery also improved hepatic steatosis and the resulting inflammation in 732 subjects evaluated by Keshishian [26]. No detrimental effects on hepatic function were noticed. Thus, in obese patients with NAFLD, gradual and substantial weight loss achieved by Roux-en-Y gastric bypass decreases hepatic fat content, inflammation, and fibrosis.

There has recently been shown to be a connection between sleep-apnea syndrome and NASH [27]. Although treatment was not studied in this paper, it raises the intriguing possibility that there may be an improvement in NAFLD secondary to treating sleep-apnea syndrome.

In summary, weight loss and physical exercise if maintained can result in an improvement in the parameters of the metabolic syndrome and an improvement in hepatic histology.

Orlistat

Orlisat, a lipase inhibitor, designed for the long-term management of obesity, decreases fat absorption, increases the excretion of the unabsorbed triglycerides and cholesterol in the stools. Together with a low-fat diet 38% of patients treated with orlistat for one year were able to lose at least 5-10% of their baseline body weight [28]. A case series of three patients with biopsy-proven NASH who were treated with orlistat for 6-12 months and who lost between 10-19 kg, showed a decrease in liver enzymes and also a decrease in steatosis, inflammation and necrosis on follow-up biopsy [29].

Recently, a study was reported from Israel of weight loss based on a 25 kcal/kg ideal body weight /day low-fat low sugar diet for 6 months [30]. 21 of these patients also received orlistat 120 mg tid. Repeat liver biopsies were performed on 23 patients at the end of the study. This treatment resulted in a decrease of liver enzymes, hepatic steatosis (from 60 to 30%) and fibrosis. However, no added benefit from the use of orlistat was noted.

Insulin-Sensitizing Agents

A prominent component of the metabolic syndrome is insulin resistance and pharmacological attempts to improve insulin-sensitivity have been examined in an effort to treat NAFLD. Work in mouse models has shown a benefit for both metformin and thiazolidinediones (glitazones) in improvement of both insulin resistance and NAFLD [31,32].

Metformin is a biguanide that down regulates hepatic glucose production and diverts fatty acids from triglyceride production to mitochondrial beta oxidation. In addition to improving insulin sensitivity and hyperinsulinemia in both animals and humans [33],

metformin also inhibits hepatic-TNFα and several TNF-inducible responses which are likely to promote hepatic steatosis and necrosis.

In a model of insulin resistance in ob/ob mice, Lin et al [31] showed that metformin significantly reduced hepatomegaly and hepatic steatosis. Marchesini et al [34] treated 14 NAFLD patients with metformin in an open label pilot study, comparing them to 6 patients who refused treatment. In addition the patients received nutritional counseling and pretreatment evaluation of insulin resistance by means of the euglycemic clamp technique and ultrasound assessment of liver volume. Treatment with metformin resulted in a significant reduction in liver volume, an improvement in insulin sensitivity and a normalization of serum aminotransferase levels in 50% of the patients. Furthermore, treatment withdrawal was associated with a return of aminotransferases to the pretreatment level.

A smaller study of 15 patients with NAFLD, proven on liver-biopsy, were treated with metformin 20 mg/kg for 1 year [35]. Although after 3 months there was a decrease in serum aminotransferases and an improvement in insulin sensitivity, there was subsequently a rise back to pre-treatment levels. A total of 10 patients had a post-treament liver biopsy and three showed an improvement in steatosis, two a decrease in the inflammation score and one an improvement in fibrosis.

More recently, the effects of metformin 850 mg bid plus dietary counseling have been compared to those of a lipid and calorie-restricted diet in an open-label study for 6 months [36]. The group given metformin (n=16) had a greater decrease in the mean serum aminotransferases levels, as well as a greater decrease in both CRP levels and insulin levels. Fifty-nine percent of the patients treated with metformin normalized serum transaminases compared to 37% in the control group (n=16). In addition there was a decrease in the index of insulin resistance as determined by the homeostasis model assessment. However, there was only a non-significant decrease in necroinflammatory activity on repeat liver biopsy at the end of treatment and no change in the fibrosis score.

There have been occasional reports of lactic acidosis following treatment with metformin. This is, however, a rare complication with an incidence rate of 9 per 100,000 patient-years according to data from 22,296 person-years of exposure [37]. In addition, a review of reports of metformin-associated lactic acidosis, found that all cases reported were associated with other contributory factors [38].

In an open-label trial, 55 non-diabetic NAFLD patients treated with 2 grams of metformin day were compared to 28 patients receiving 800IU of vitamin E per day and 27 patients treated by a prescriptive weight-reducing diet [39]. Metformin treatment was associated with higher rates of aminotransferase normalization, after correction for age, gender, basal aminotransferases, and change in body mass index compared to both control groups. In addition, in seventeen metformin-treated cases that had a rebiopsy done, there was a significant improvement in liver fat, inflammation and fibrosis (figure 1).

Recently, Blaszyk and colleagues [40] treated 10 patients with biopsy-proven NASH with a 48-week course of metformin (2 g/day). Metformin improved hepatic necroinflammation but did not improve hepatic fibrosis.

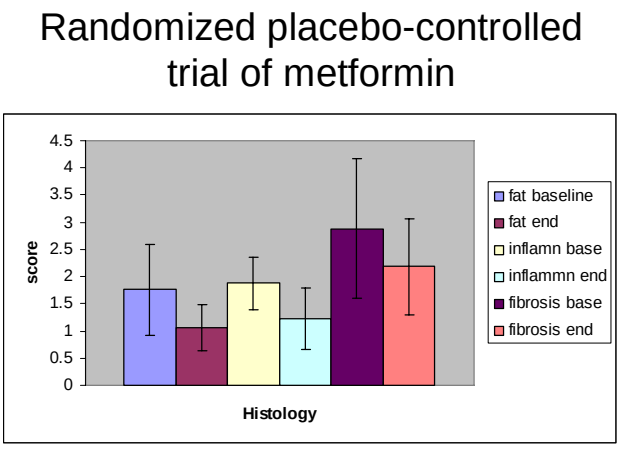

Figure 1. The effect of metformin on histological parameters of NAFLD. 55 patients with NAFLD were treated with 2 grams of metformin per day for 12 months. There was a significant decrease in both the degree of steatohepatitis and fibrosis in the 17 patients who had a follow up biopsy (Bugianesi E et al [39]).

In summary, there may be a benefit of metformin in the treatment of patients with NAFLD although the evidence is inconsistent. This needs to be resolved by further randomized controlled trials.

Thiazolidinediones

This novel class of drugs improve insulin sensitivity by acting as ligands for the peroxisomal proliferators activated receptor (PPAR) γ class of nuclear transcription factors [41]. Caldwell et al [42] treated ten patients with the first clinically available medication in this class troglitazone for up to 6 months. Seven of the ten patients in the study achieved a normalization of serum aminotransferases but there was no histological response. Subsequently, trogliazone was withdrawn from the market due to idiosyncratic and severe hepatotoxicity [43].

There are now second-generation thiazolidinediones on the market- pioglitazone and rosiglitazone. They appear to have a safer hepatic profile than troglitazone.

Rosiglitazone has been tested on 30 patients with NASH, 8 of whom had diabetes, in a open-label study. The treatment was for 48 weeks in a dose of 4 mg bid but the interim results were published after 24 weeks [44]. At 24 weeks there was no improvement in insulin sensitivity although there was reduced liver fat content (estimated by CT scanning). There was a mean weight gain of 3.5%. The data from the longer term follow up have confirmed the previous data and included data on posttreatment biopsies in 26 patients. There was a significant improvement in mean global necroinflammatory score, hepatocellular ballooning and zone 3 fibrosis. Ten patients (45%) no longer met the criteria for NASH. Disturbingly, the weight gain continued to increase and following 48 weeks of treatment there was a mean increase of 7.3% [45] (Figure 3).

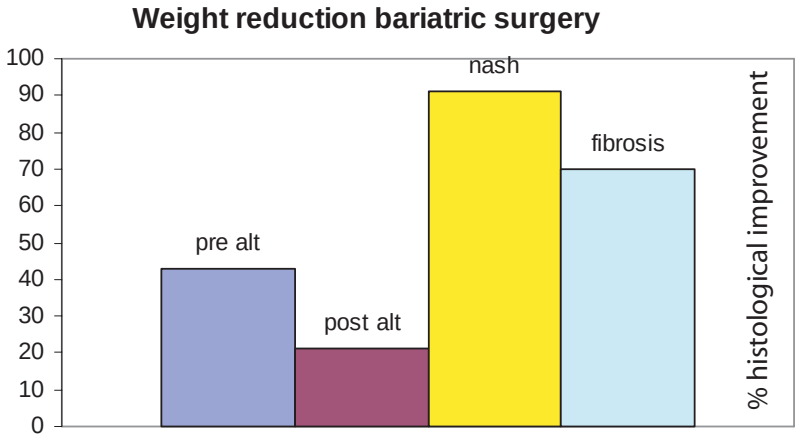

Figure 2. Weight reduction with bariatric surgery. Repeat biopsy after a mean of 25.6±11.0 months (n=36). There is a decrease in the alanine aminotransferase level (alt) as well as improvement in steatohepatitis (nash) and fibrosis scores (Dixon et al [18]).

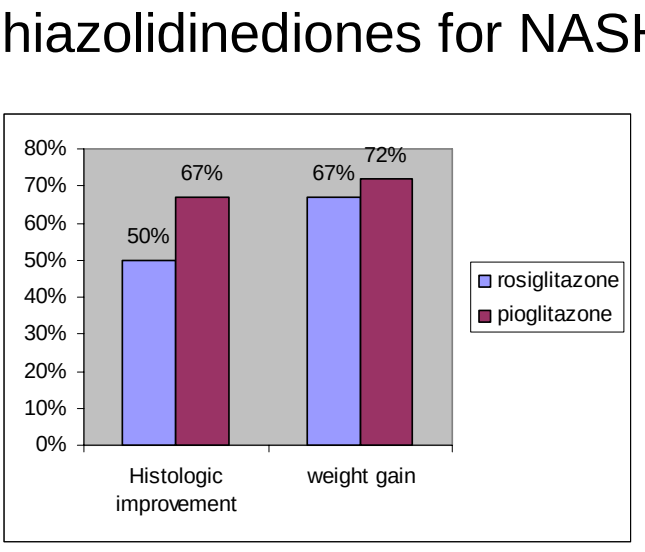

Figure 3. Effect of treatment with thiazolidinediones. Two randomized placebo-controlled trials on patients with NASH. Rosiglitazone – 4mg bid for 48 weeks (n=30) [45]; Pioglitazone – 30 mg per day for 48 weeks (n=18) [46].

Pioglitazone in a dose of 30 mg daily has been examined in a pilot study of 18 non-diabetic patients, without a control group, from the NIH [46]. After 48 weeks of treatment, serum alanine aminotransferase levels fell to normal in 72% (figure 3). In addition, there was a decrease in hepatic fat content and size as assessed by magnetic resonance imaging, as well as a reduction of fasting glucose, insulin and free fatty acids, indicating improved insulin sensitivity. In this study there was a significant improvement in steatosis, cellular injury, parenchymal inflammation, Mallory bodies and fibrosis. There was however, a side effect of weight gain (average of 4%) and an increase in total body adiposity (Figure 2). Despite these favorable results, the long-term effect of pioglitazone remains to be determined. It is possible

that continued therapy might result in continued weight gain, which may reverse any potential beneficial effects. The same group has recently reported in abstract form, the results of follow-up of 21 patients who discontinued pioglitazone. In these patients, a return of insulin resistance, increase in serum ALT levels, and a worsening of hepatic steatosis and inflammation was noted [47].

Harrison and colleagues [48] performed a randomized, double-blind, placebo-controlled trial to examine the efficacy of pioglitazone (45 mg daily for 6 months), in 22 patients with biopsy-proven NASH. Treatment with pioglitazone resulted in an approximately 2.5-fold increase in plasma adiponectin, reduced ALT levels, and a 25% reduction in hepatic fat content by magnetic resonance imaging. A significant improvement in ballooning degeneration, steatosis, and fibrosis was only seen with pioglitazone treatment.

Sanyal et al [49] recently reported a randomized controlled prospective study comparing 30 mg of pioglitazone and 400 IU of vitamin E to 400 IU of vitamin E alone for 6 months. There were ten patients in each arm. The combination treatment produced a decrease in steatosis, cytologic ballooning, Mallory's hyaline and pericellular fibrosis compared to the vitamin E monotherapy arm.

A potential concern is subclinical cardiac failure [50], which is a risk in hypertensive patients with the metabolic syndrome. In addition there have already appeared in the literature post-marketing case reports of rosiglitazone- and pioglitazone-induced liver injury, as well as cholestatic jaundice [51].

Lipid-Lowering Agents

One of the central elements of the metabolic syndrome is hyperlipidemia, with high levels of cholesterol, triglycerides and LDL-cholesterol and low levels of HDL-cholesterol. This is the basis for the use of lipid-lowering agents as treatment for NASH.

Two small studies have evaluated the effects of fibrates in NAFLD. Clofibrate in an open label pilot study at a dose of 2 grams per day for one year did not produce any significant change from baseline in either enzyme levels or histology [52]. Gemfibrozil, however, was shown to be more effective than diet in reducing aminotransferase levels, irrespective of baseline triglyceride levels [53). This open label study lasted however for only 4 weeks.

In a report of only 2 patients treated with tamoxifen, bezafibrate prevented the histological progression of NASH, although this was secondary NASH [54].

There are only a few reports on the use of statins for treatment of NASH. There has been reluctance to administer statins to patients with any preexisting liver disease, but a recent review of the literature has not found strong evidence for liver damage [55]. In addition the levels of transaminases in a group of patients with NAFLD decreased with a treatment program including statins [56].

In one study 20 mg of pravastatin was administered to five patients with NASH for 6 months and the hepatic histology was reexamined in 4 patients [57]. The serum transaminases were normalized in all 5 patients and there was an improvement in both steatosis and hepatic inflammation. In another study, atorvastatin was administered to seven patients and the hepatic histology rechecked after a mean period of 21 ± 2 months [58]. There was no

significant increase in liver enzymes although in some cases, an improvement of hepatic histology was noted. Kiyici et al administered 10 mg per day of atorvastatin for 6 months to 27 patients with biopsy-confirmed NASH. Liver density, assessed by CT scan, was found to decrease presumably due to a decrease in fat content [59].

Thus at present there is not conclusive evidence for a beneficial effect of statins in NAFLD and further evidence in the form of randomized controlled trials are required.

Probucol

Probucol is a lipid-lowering agent with anti-oxidant properties. Thirty cases of biopsy-proven NASH were randomly allocated to 500 mg of procubol daily for 6 months (n=20) or placebo (n=10). There was a significant decrease in the serum transaminases in the treatment group compared to the placebo group and nine patents in the treatment group normalized their transaminases, compared to none in the control group [60].

Anti-Oxidants

Vitamin E is an antioxidant [61] and this has prompted examination of its effect on NAFLD. In an uncontrolled trial of eleven children with NASH, supplementation with vitamin E in a dose of 400 to 1200 IU daily was found to produce a decrease in serum transaminases, which was reversible on cessation of the therapy. There was no change in liver echogenicity on ultrasound and histology was not examined in this study [62].

A randomized, placebo-controlled trial of 45 patients included 22 patients treated with both vitamin C (1,000 mg) and vitamin E (1,000 IU) for 6 months [63]. In this study there was a significant improvement in fibrosis scores in the NASH patients receiving the vitamins compared to baseline but there was no change in the necroinflammatory score or ALT. However, the histological improvement was not significantly different from the improvement seen in the placebo group.

Vitamin E supplementation was found to offer no benefit over lifestyle modifications in a study involving 16 patients [64]. The lifestyle modifications consisted of a step 1 American Heart Association diet plus aerobic exercise with or without the addition of 800 units of vitamin E per day. The end-point was a decrease in serum transaminases.

The efficacy of pioglitazone plus vitamin E was compared in a pilot study of ten patients receiving 400 IU of vitamin E per day and 10 patients receiving 400 IU of vitamin E and 30 mg of pioglitazone per day for 6 months [65]. Treatment with vitamin E alone resulted in a significant decrease in steatosis, whereas the combination therapy resulted in a decrease in steatosis, cytologic ballooning, Mallory's hyaline and pericellular fibrosis.

A recent study of the effect of metformin in an open-label randomized trial of non-diabetic patients with NAFLD compared the effect to a control group of a prescriptive weight-reducing diet and another group of 28 patients given 800 IU of vitamin E alone. There was no significant effect of vitamin E in terms of ALT levels and metabolic parameters [39].

Betaine is a naturally occurring metabolite of choline and raises S-adenosyl methionine levels that may play a role in decreasing hepatic steatosis. In a pilot study [66] of ten adult patients, 7 of whom completed a year of therapy with betaine, there was an improvement in serum transaminases and also an improvement in the degree of steatosis, necroinflammaory grade and stage of fibrosis. A larger randomized trial (191 patients) compared treatment with

betaine and diethanolamine gluconate and nicotinamide ascorbate to placebo. There was a significant decrease of 25% in hepatic steatosis and 6% in hepatomegaly [67].

Ursodeoxycholic Acid (UDCA)

This hydrophilic bile acid has hepatoprotective properties and is the treatment of choice for primary biliary cirrhosis [68]. A pilot study of 24 patients who received 12 months of UDCA in a dose of 13-15 mg/kg/day showed a decrease in liver enzymes and hepatic steatosis on biopsy [52]. An improvement in liver enzymes was also found in a study in 17 normolipidemic NASH patients [69].

However, a randomized, placebo-controlled trial (level 1c evidence) of 13-15 mg/kg/day of UDCA for 2 years in a total of 126 patients found no difference in either liver biochemistries or histology compared to controls [70]. The possible reasons for the negative result have been elegantly stated in an accompanying editorial [71], including statistical underpowering, heterogeneity of biopsy, variability of liver enzymes and regression to the mean.

A subsequent double-blind placebo-controlled trial of 14 women with a BMI of greater than 27 kg/m^2 who were treated with a 12000 kcal/day diet and 1200 mg of ursodeoxycholic acid and compared to 13 women with a similar BMI who were treated with just the 1200 kcal/day diet, showed a similar reduction in BMI, serum transaminases and hepatic steatosis index determined by ultrasound [72].

Another randomized, placebo-controlled double-blind study of urodeoxycholic acid (10 mg/kg/day) for 3 months in the absence of weight loss resulted in a decrease in serum transaminases but no change in hepatic fat content as assessed by CT [73].

Recently, the combination of UDCA and 800 IU of vitamin E per day has been shown to produce a significant decrease in both liver transaminases and steatosis, activity index and fibrosis compared to both placebo and UDCA alone. This paper has been reported in abstract form only [74].

Angiotensin II Receptor Antagonists

Angiotensin II has been shown to play a role in hepatic fibrosis and in rats an angiotensin II type 1 receptor antagonist has been shown to decrease hepatic fibrosis [75]. A pilot study of 50 mg of losartan per day in seven patients with both NASH and hypertension has been shown to decrease serum aminotransferases, decrease hepatic necroinflammation (5/7) and reduce hepatic fibrosis (in four out of seven patients) [76]. There was no change in the degree of lobular steatosis.

Pentoxifyline

Tumor necrosis factor-alpha (TNF-α) is thought to play a role in the development of insulin resistance central to the metabolic syndrome and also to have a role in the progression of NAFLD through both inflammatory, apoptotic and fibrotic mechanisms [77].

An open-label trial of 20 patients with biopsy-proven NASH given pentoxifyline 400 mg qid for 12 months resulted in a significant decline in serum transaminases but not alkaline phosphatase or bilirubin. Of the 20 patients 9 withdrew due to nausea [78]. In another study, 18 patients with biopsy-proven NASH were treated with pentoxifyline 400 mg tid for 6

months [79]. There was some improvement in metabolic parameters despite the fact that there was no weight loss. In addition the serum transaminases decreased and were normal in 60% after 6 months of treatment. In addition there was a significant decrease in fatigue.

Phlebotomy

There is some evidence linking NASH to elevated serum ferritin and iron concentration [80]. Hyperferritinemia is, however, a marker of systemic inflammation rather than a marker of increased iron body content [81]. It is possible that increased hepatic iron and excessive fat accumulation may be involved in the second hit necessary for steatohepatitis and fibrosis [82]. In addition iron accumulation may induce insulin resistance [83] and iron removal by venesection may reduce this insulin resistance [84].

Facchini et al [85] caused iron depletion in 42 carbohydrate-intolerant patients who were free of the 2 common hemochromatosis mutations –C282Y and H63D, and who had a serum iron saturation lower than 50%. In 17 of these patients who had NAFLD, there were normal levels of body iron, but following iron depletion there was an improvement in both fasting and glucose-stimulated plasma insulin concentrations and a decrease in serum ALT levels.

More recently, a study on 25 patients with NASH found no hepatic parenchymal iron overload on Prussian blue staining. The authors suggest that iron overload may be a result of hemochromatosis gene mutations and that their results may be due to the lower frequency of the HFE mutations in Turkey [86]. A similar lack of an association between hepatic iron accumulation and NASH has been reported by another group from Turkey [87] and Brazil [88].

Leptin

Leptin deficiency or resistance results in steatosis [89] Lipodystrophy is a rare condition associated with an absence of adipose tissue and resultant leptin deficiency. The liver acts as a major storage site for triglycerides in such patients and may develop NASH [90]. In a study of eight patients with lipodystrophy and two patients with Dunnigan's partial lipodystrophy, eight had histological criteria for NASH on a baseline liver biopsy [91]. Treatment with recombinant methionyl human leptin (r-metHuLeptin), was given for a mean duration 6.6 months and repeat histological examinations showed significant improvements in steatosis and ballooning injury together, with a reduction of mean NASH activity by 60%. There was no change in the fibrosis score. In addition there was also a significant decrease in both serum transaminases, triglycerides and liver volume. It is unclear what impact leptin may have in other patient groups with NASH but use of leptin in obese patients to date has been disappointing.

Probiotics

It has been suggested that gut-derived endotoxemia may contribute to the evolution of both alcoholic and nonalcoholic steatosis, fibrosis and portal hypertension [77]. Oral antibiotics that are poorly absorbed or administration of lactobacilli have been shown to inhibit the progression of steatosis to steatohepatitis in animals with obesity or animlas fed alcohol and also to improve the hemodynamics of portal circulation in patients with portal hypertension [92-4]. Loguercio et al have recently reported a open label pilot study of the use

of the probiotic VSL#3 in 22 patients with biopsy-proven NAFLD for 3 months. This preparation contains 450 billion bacteria of different strains and has improved fatty liver in experimental animals [93]. There was a decrease in serum aminotransferases, increase in albumin and a decrease in the markers of lipid peroxidation malondialdehyde and 4-hydroxynoneal [95]. This pilot study is of a very short duration and there is a limited amount of data available. Further investigation in the form of a randomized controlled trial is necessary.

Liver Transplantation

A subgroup of patients with advanced NASH develop end-stage liver disease and require liver transplantation. This is often complicated by the presence of comorbid conditions related to diabetes, obesity and hyperlipidemia. In addition receurrence of the NASH in the transplanted liver has been reported [96-9].

SIDE-EFFECTS OF TREATMENT AND COST EFFECTIVENESS

There is a lack of level 1 data from randomized controlled trials with end-points such as mortality or quality of life on which to base therapeutic decisions. Indeed the natural history of NAFLD is only just becoming apparent [100]. A report from the Mayo Clinic of a survey of community-diagnosed NAFLD patients in a population-based cohort from Olmstead County with a mean follow-up of 7.6 years found an increase in mortality associated with age, impaired fasting glucose and cirrhosis. Liver disease was the third leading cause of death. In this study, 71% were obese, 26% had diabetes and 68% hypertriglyceridemia. This demonstrates that lifestyle changes will be relevant for the vast majority of NAFLD patients.

Table 2. Summary of trials with thiazolidinediones.

Name	number	Level of evidence	time	Parameters examined	biopsy
Caldwell et al.	10	2b	6 months	Transaminases decrease	no improvement
Neuschwander-Tetri et al.	30	2b	48 weeks	Transaminases decrease Liver fat decreased on CT	improvement
Shadid et al.	3	2b	18 weeks	Decreased transaminases	nd
Promrat et al.	18	2b	48 weeks	Decreased transaminases Decreased hepatic fat on MRI	improvement

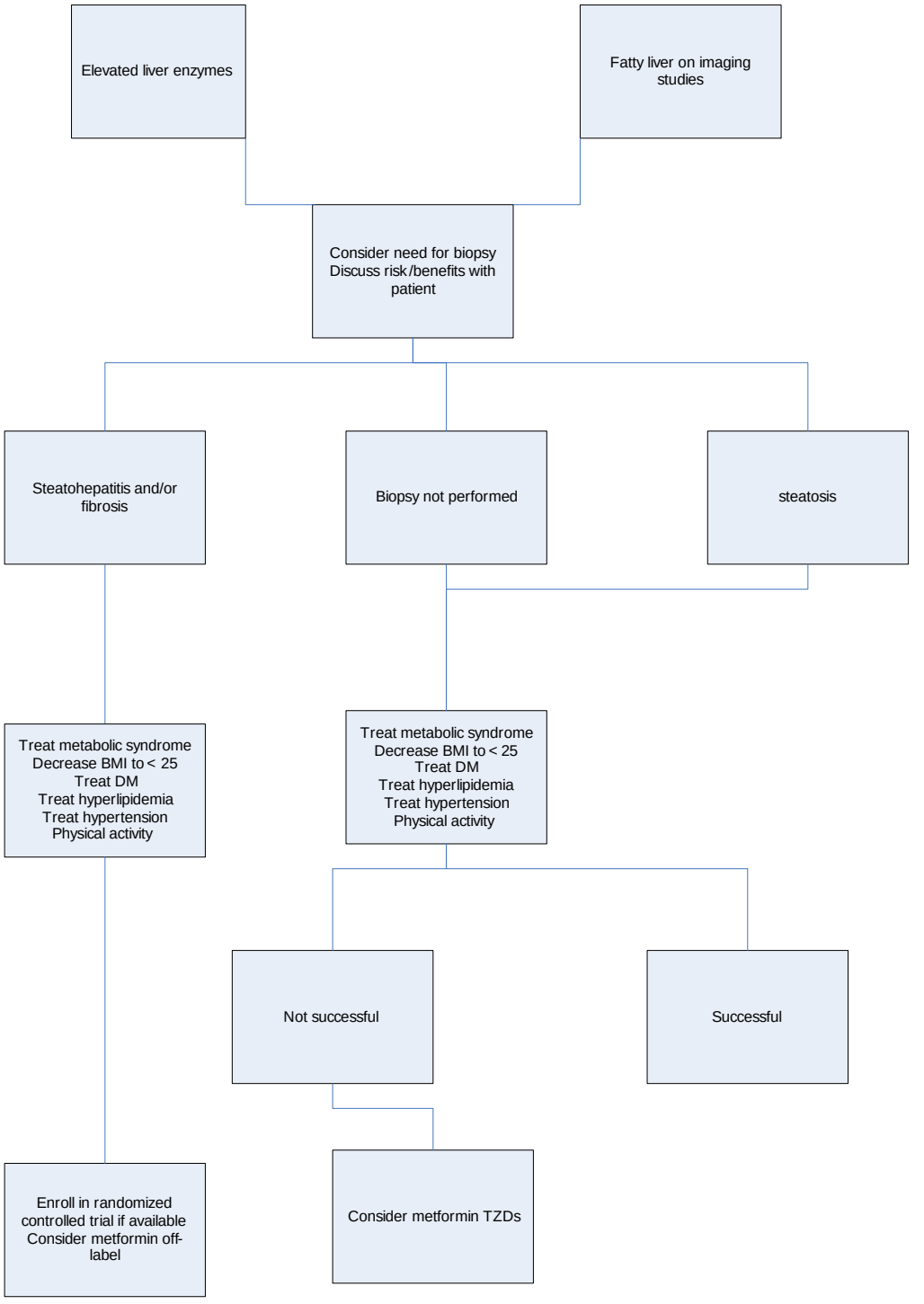

Figure 4. Suggested therapeutic approach to patients with NAFLD.

CONCLUSION

In this chapter we have reviewed the current literature regarding the treatment of NAFLD. In order to conclude we will examine the 4 principles of treatment noted in the beginning.

1. The natural history of the disease is becoming clearer and it is apparent that there is a significant morbidity and mortality associated with NAFLD.
2. At present liver biopsy is required in order to differentiate those patients with a benign disease from those in whom there is going to be progression. The indications for liver biopsy and the role of non-invasive tests fro fibrosis and inflammation are still unclear.
3. The majority of evidence available, showing positive results including improvement of the surrogate end-point of histology, are derived from studies that evaluated weight loss and lifestyle changes. Since such an intervention is cheap and also clearly effective in reducing cardiovascular risk factors, this is the treatment of choice. The exact role for bariatric surgery needs to be defined. Medical therapy for NAFLD is still evolving and there is a need for large randomized controlled trials.
4. The only proven cost-effective treatment at this stage is weight loss. The benefit-risk ratio of other treatments needs to be established.

In the absence of such information, our current recommendation is to adequately address the components of the metabolic syndrome that are the main risk factors. The treatment needs to be individualized for each particular patient. The approach for a 75 year old is not going to be the same as for a 25 year old. Our recommendation is shown in figure 4.

There is evidence to suggest a histological improvement in NAFLD following weight loss. In addition the overall benefits of losing weight, increasing physical activity, controlling hypertension, hyperlipidemia and diabetes mellitus are well described [101].

The challenge for clinical hepatologists is to perform well-designed clinical trials with a high power to detect clinically relevant end-points on which future therapeutic interventions can be based.

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Chapter IV

THE HEPATITIS C VIRUS AND DIABETES MELLITUS ASSOCIATION: CHARACTERIZATION AND STUDIES OF RISK FACTORS, MECHANISMS, IMPLICATIONS AND TREATMENT

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ABSTRACT

Chronic hepatitis C virus (HCV) infection is a multifaceted disease with extra hepatic manifestations. The link between HCV and type 2 diabetes mellitus (DM) was described more than a decade ago but only recently its importance has been recognized. Several studies provided compelling evidences that chronic HCV is specifically and frequently associated with diabetes, regardless of the presence of liver cirrhosis. Diabetes and glucose intolerance occur in more than a third of HCV patients and the underlying mechanism is insulin resistance which occurs early in the course of the disease. The two major types of risk factors for developing HCV associated DM relate either to a more severe hepatic histology or to the presence of 'traditional' risk factors for type 2 DM such as age, obesity and positive family history of diabetes. The mechanisms by which HCV leads to insulin resistance are still elusive. We and others provide intriguing data suggesting that activation of tumor necrosis factor (TNF)-α has a pivotal role in the HCV-DM association. However other direct and indirect effects of HCV on the insulin signaling cascade can not be ruled out. The implications of this extra hepatic involvement are immense and relate both to the complications of diabetes as well as to an unfavorable course of the hepatic disease with poor response to antiviral therapy, observed in HCV

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patients with insulin resistance. Future studies are needed to evaluate the role of antiviral treatments as well as insulin sensitizing agents in improving both glucose tolerance and the course of the liver disease.

HEPATITIS C

Since its discovery in 1989 [1], hepatitis C virus (HCV) and chronic hepatitis C have been established as a health problem of worldwide distribution and immense proportions. It is estimated that about 170 to 200 million people are chronically infected with the virus. In the USA, 1.8% of a random sample of the population test positive for anti-HCV, while in parts of Eastern Europe and Africa prevalence rates may approach 15% in some countries [2,3]. HCV is an RNA virus which is transmitted predominantly through infected blood and although the acute infection is hardly ever felt - it becomes chronic in 85-90% of infected individuals. All patients develop features of chronic hepatitis which is characteristically indolent for a few decades and may be barely symptomatic, often without even raised serum aminotransferase levels. Nevertheless, cirrhosis develops in as many as 1 in 5 patients and hepatocellular carcinoma (HCC) is another dreaded outcome [3,4]. Combination treatment with pegylated interferon alpha and ribavirin over 48 weeks is currently the best option for chronic hepatitis C patients [5]. However, treatment is costly, may be poorly tolerated and sustained virological response can be attained by less than half of treated patients.

'EXTRA-HEPATIC' MANIFESTATIONS

Chronic HCV infection is often associated with varied extra-hepatic manifestations which have been well studied. The presence in the serum of immunoglobulins that precipitate below body core temperature ("cryoglobulins") can be detected in over 50% of HCV-infected individuals and diverse autoantibodies or monoclonal gammopathies can also be frequently found [6-8]. These are mostly asymptomatic however. Overt clinical syndromes are less common. They include mixed cryoglobulinemia – a systemic vasculitis secondary to circulating immune complex deposition in small vessels which occurs in about 5% [8]; immune thrombocytopenia [9]; thyroid disorders; membranoproliferative glomerulonephritis; porphyria cutanea tarda; lichen planus; Sjogren's syndrome; Mooren's corneal ulcers; polyarthritis; anti-LKM-positive autoimmune hepatitis and the development of B-cell malignant lymphomas. The exact mechanism responsible for these varied associated disorders remains elusive, but HCV is a lymphotropic as well as hepatotropic virus and expansion of autoantibody-producing B-cells in chronic HCV infection appears central to the pathogenesis of these disorders which are all immune-mediated [10].

DIABETES AND HCV: FIRST OBSERVATIONS

Can diabetes be the most common disease associated with chronic hepatitis C? Surprisingly, the link was hardly noticed at first, despite the extensive research on HCV and the large number of patients affected. A higher incidence of diabetes in liver transplant recipients with hepatitis C was noted by us at the Mount Sinai Medical Center, New York and reported to the American Diabetes Association (ADA) meeting in 1993 [11]. Post transplantation diabetes mellitus (PTDM) occurred in as many as 8/13 (62%) of patients whose liver failure was HCV-related, vs. 3/34 patients (9%) with other causes of liver failure ($P<0.001$) [12]. Thus, in addition to the known hyperglycemic effects of immunosuppressive drugs, chronic hepatitis C was suggested as an independent risk factor for the development of PTDM [13,14]. Allison et al. from Cambridge conducted in 1994 a retrospective study of diabetes among adult patients with cirrhosis who underwent liver transplantation. Fifty percent (17/34) of patients with chronic HCV infection had diabetes vs. none of the patients who had alcoholic cirrhosis or hepatitis B virus (HBV)-related liver disease [15]. These initial reports involved special groups of patients who were prone to altered glucose metabolism due to liver cirrhosis [16] or transplantation. However, the suggested link between HCV and diabetes was further supported by brief reports from Italy and Turkey. Taliani at al. found the prevalence of diabetes mellitus (DM) to be 18.7% among patients with chronic HCV infection [17] and this observation was confirmed in 1996 and shown to be significantly different compared to HBV infection [18-20]. Conversely, when diabetic patients were evaluated, an increased prevalence of anti-HCV antibodies was found [21], especially if the diabetic patients had abnormal liver function tests [22,23]. A later study found no significant difference for HBsAg seropositivity between type 2 diabetic patients and controls. In contrast, 7.5% of 692 diabetics were anti-HCV positive vs. 0.1% only of over a thousand healthy blood donors [24]. These initial observations set the stage for further research that firmly established the association between chronic hepatitis C and diabetes and later moved on to try and elucidate its mechanism.

DIABETES MELLITUS IN CHRONIC HEPATITIS C

Liver cirrhosis is strongly associated with glucose intolerance. As many as 70-80% of patients with cirrhosis have impaired glucose tolerance and 10-20% of cirrhotic patients are known to have diabetes [25-27]. In one recent study, diabetes was present in 32.3% of 247 patients with cirrhosis [28]. Therefore, the finding that 21-50% (median 26%) of 956 HCV-infected patients studied had diabetes [15,19,20,29,30] – significantly more than the prevalence of diabetes in other chronic liver diseases including hepatitis B, needed to be reaffirmed by analyzing patients who were *definitely without liver cirrhosis.*

We have studied 45 consecutive patients with chronic hepatitis C in whom cirrhosis was excluded by clinical, laboratory, technetium 99 liver-spleen scintigraphy and liver biopsy. Other possible etiologies of liver disease (such as alcohol consumption) were exclusion criteria and additional patients with chronic HBV infection (n=88) and healthy individuals (n=90) were studied as control groups. We found that as many as 15/45 HCV patients were

diabetic (33%), as compared to 12% in the HBV group and 5.6% of the healthy matched controls [31]. The diagnosis of HCV preceded the diagnosis of diabetes in 11/15 patients and the diabetes required insulin treatment in 1/15 patients only. Comparing the groups of HCV patients with and without diabetes (Table 1) we found that a family history of diabetes was common in the HCV/DM patients (P<0.001). Comparing the patients' biochemical and histological parameters, we found that the diabetic HCV patients had a trend for higher liver enzymes and importantly, they had significantly higher inflammatory activity, more fibrosis and more steatosis in their liver biopsies compared to patients with chronic hepatitis C who had no diabetes (Table 2).

Table 1. Clinical characteristics of 45 chronic hepatitis C patients, with and without diabetes (mean values ± SD).

	Nondiabetic (n=30)	Diabetic (n=15)	P
Age, years	51.3±10	54±14	NS
Male/female	12/18	5/10	NS
Duration of HCV (months)	54±26	56±31	NS
BMI (kg/m^2)	26±3	27±5	NS
Family history of diabetes	2/30 (7%)	10/15 (67%)	p<0.0001
HCV genotype:#			
1b	10 (53%)	9 (90%)	p < 0.05
1a	4	-	
2	4	1	
3	1	-	
Interferon treatment, %	80	87	NS

29 patients were studied; NS = Non significant; BMI= Body mass index.

Two notable large cohort studies strongly support our findings. *First*, the large ongoing National Health and Nutrition Examination Survey (NHANES III) evaluated 9841 community-living subjects of whom 8.4% had type 2 DM and 2.1% were anti-HCV positive. Analysis showed that persons 40 years of age or older who were anti-HCV positive, had an adjusted odds ratio of 3.77 (95% CI, 1.80-7.87) for type 2 DM [32]. As previously noted, this finding was adjusted for possible confounding factors such as sex, body mass index (BMI), ethnicity, poverty index, and previous drug or alcohol use and was not found in hepatitis B infection. The HCV positive group had no clinical stigma of chronic liver disease, although no liver biopsies data were available [33]. *Second*, more recently, in a large cohort of consecutive patients with chronic hepatitis C in Spain, a threefold increase in the prevalence of glucose abnormalities was observed compared with HCV-negative subjects [34]. In fact, 32% of 380 patients had either diabetes or impaired fasting glucose (IFG) (about 1:1 ratio). Moreover, multivariate analysis of patients with chronic hepatitis without cirrhosis, found HCV infection to be an independent predictor of glucose abnormalities with an odds ratio of 4.26 (95%CI 2.03-8.93). This study is notable, since it clearly demonstrates a) that for every patient with chronic hepatitis C and diabetes, another patient already has impaired fasting glucose (fasting blood glucose between 110 and 125 mg/dl); and b) that standard 2-hr 75g

oral glucose tolerance test (OGTT) in HCV patients with chronic hepatitis who have no diabetes may reveal either impaired glucose tolerance or diagnose unsuspected diabetes in a substantial number of patients (15/50 and 9/50, respectively) [34]. Essentially similar results in non-cirrhotic HCV-positive patients have been reported in 2005 from Italy [35] and also from the Mayo Clinic [36]. The prevalence of diabetes mellitus and IFG were significantly higher among patients with chronic hepatitis C than controls and patients with advanced vs. early liver histology were at a greater risk of diabetes [36], supporting our own observations [31]. Thus, chronic hepatitis C is *specifically and frequently* associated with diabetes, regardless of the presence of liver cirrhosis. In many additional patients who do not fulfill the ADA criteria for diabetes, impaired glucose tolerance is already present indicating possible prediabetes.

Table 2. Biochemical and histological parameters of 45 chronic hepatitis C patients, with and without diabetes (mean values ± SD).

	Nondiabetic (n=30)	Diabetic (n=15)	P
Laboratory values:			
AST (max), U/L^{-1}	87±54	129±135	NS
ALT (max), U/L^{-1}	124±74	196±219	NS
γGT (max), U/L^{-1}	70±71	101±89	NS
Albumin, g/L^{-1}	45±0.3	44±0.4	NS
Liver biopsy findings:			
Hepatitis activity index*	8.6±3.6	11.6±3.9	p<0.02
Fibrosis (%)*	1.0±0.8	2.0±1.0	p<0.001
Steatosis (%)**	7.2±11.0	20.3±15.4	p<0.002

***** Inflammation and fibrosis graded according to Knodell score (Knodell RG, Ishak KG, Black WC, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981; 5:431-5).

****** Steatosis (percentage of cells with fatty changes) may be secondary to diabetes.

NS = Non significant; AST= Aspartate aminotransferase;
ALT= Alanine aminotransferase; γGT= γ-Glutamyltransferase

RISK FACTORS FOR THE HCV-DM ASSOCIATION

The association identified between HCV infection and diabetes was considered intriguing and important, as evidenced by the many editorials that were devoted to it since 1996 [37-42]. This led to increasing research efforts by several groups, yielding ever more data for analysis. As a result, several risk factors for the HCV-DM association have emerged, and other variables were not found to affect the risk of developing diabetes. A careful study of relevant risk factors may be an indicator of the mechanism of the association and thus it may be of considerable importance.

In the Atherosclerosis Risk in Communities (ARIC) Study, a 9-year follow-up showed that antecedent HCV infection was a significant risk factor for developing diabetes in patients

with advanced age or high BMI, with a remarkable relative hazard of 11.58 (95 CI, 1.39-96.6) [43]. Other risk factors for developing diabetes in HCV patients include positive family history of diabetes and black race, but not the presence in serum of autoantibodies characteristic of type 1 DM [30-32]. Thus, HCV leads to type 2 DM particularly *in susceptible hosts*. How is this susceptibility acquired? Additional risk factors have been recently investigated and this may prove important in deciphering the pathogenesis.

Table 3. Factors affecting risk of diabetes mellitus in patients with chronic hepatitis C virus infection*.

Associated with an increased risk of diabetes	
@ **Age ≥ 40**	[19, 30, 32, 51, 56, 57]
@ **BMI**, increased	[32, 51, 52]
@ **Family history** of diabetes	[31, 36, 51]
@ **Black** ethnicity	[58]
@ **Liver enzyme**, higher levels (serum aminotransferases)	[31, 50, 54]
@ **Hepatic histology**, more adverse	[36]
Inflammation (HAI)	[31, 52, 54]
Fibrosis	[31, 49, 51, 54]
Steatosis	[31, 51, 57, 59, 60]
@ **Cirrhosis**, relative to no cirrhosis Child-Pugh score, increased	[56, 28]
@ **Serum ferritin**, increased levels	[61]
@ **TNF-alpha system**, activation	[55]
Not** associated with increased risk of type 2 diabetes	
# Autoantibodies to insulin or islet cells	[30, 31, 46, 47]
# Interleukin-6	[55]
# Interferon treatment***	[48]
*May **ameliorate** the risk of diabetes in chronic hepatitis C*	
• 'Traditional' lifestyle modification	Under investigation
• Insulin sensitizing agents	Under investigation
• Interferon therapy?	[50, 52, 62]
• Anti-TNF agents	Under investigation

* Chronic hepatitis B does not confer a similarly increased risk (see text).

** Results concerning the effect of *male gender* [30,32], liver *iron deposition* and *viral load* [51,62- 64] remain controversial. High HCV core titer was reported to increase risk of diabetes [65]. Conflicting results have also been reported regarding the possible effect of HCV *genotype* [30,31,34,51,52,63] and it is hard to determine at present whether the genotype of the virus alters susceptibility to diabetes or not.

*** Rarely *diabetes type 1* may develop [48].

Interferon treatment may often be associated with the development or exacerbation of autoimmunity in animal models and humans [44], including in patients with chronic hepatitis C [45] who may be more susceptible than others [10]. Indeed, interferon therapy is often implicated in the literature as having a role in the development of diabetes in HCV patients.

However, this association is rare. The vast majority of HCV patients treated with interferon, do not exhibit increased frequency of clinical or latent autoimmune diseases [46,47] and the few reported cases of DM developing during interferon therapy, had developed type 1 DM [48] unlike the diabetes reported in most patients with HCV infection. Konrad et al. of Frankfurt who studied glucose tolerance and insulin sensitivity in HCV patients before and after therapy with interferon-alpha, found no evidence of interferon-related impairment of glucose homeostasis [49,50].

Thus, no evidence of β-cell-directed autoimmunity was found in HCV/DM patients [30,31,47]. In contrast, there is substantial evidence to establish that patients with chronic hepatitis C and diabetes are *insulin resistant* and that insulin resistance (IR) develops *early* in the course of the infection [51-53]. Petit et al. of Dijon, France conducted an elegant study of 123 consecutive untreated chronic hepatitis C patients, 13% of whom were diabetic. In addition to showing that older age, obesity and family history of diabetes increase the risk of diabetes in HCV, their study also reveals the central importance of liver fibrosis [51]. Moreover, when insulin resistance assessed by the homeostasis model assessment (HOMA-IR) was determined for 81 of the 107 non-diabetic patients, a higher grading of fibrosis was independently related to insulin resistance, strongly supporting liver fibrosis as an important risk factor for the HCV-DM association and also establishing that IR already occurs at an early stage in the course of HCV infection, long before the appearance of cirrhosis. This relationship between severity of the hepatitis and impaired glucose tolerance in noncirrhotic patients was observed by us in 1999 [31] and confirmed by Konrad et al. [49,54], Zein et al., [36] as well as by our own group in our next study [55]. It may be concluded that HCV patients who develop diabetes have a more severe liver disease according to both their liver enzymes and biopsy findings [31,51,54]. Furthermore, insulin sensitivity in nondiabetic HCV patients is significantly correlated with serum aspartate aminotranferase, histological activity index and the degree of fibrosis [54]. A recent study of 260 HCV-infected patients confirmed that insulin resistance was an independent predictor of the degree of fibrosis [52]. The studies on the various risk factors for diabetes in HCV infection are summarized in Table 3 which also shows factors that were examined and found *not* to affect diabetes risk in HCV or those that may possibly ameliorate this risk [56-65]. As the Table reveals, the two major types of risk factors for developing diabetes in HCV relate either to *a more severe hepatic inflammation and worse histology* or to the *presence of 'traditional' risk factors for type 2 DM*. The more factors present – the higher the patient's risk. The effects of obesity and ageing for example, on insulin sensitivity are well known and not unique of course, to chronic HCV infection. The question remains however, how can aggravated HCV-induced liver inflammation and fibrosis be linked to insulin resistance?

PATHOGENESIS OF THE HEPATITIS C ASSOCIATED DIABETES

As discussed in previous sections, the HCV-associated diabetes has the characteristics of type 2 DM. Insulin resistance (IR), is known to have a pivotal role in the pathogenesis of type 2 DM, and most studies evaluating insulin action in HCV patients found evidences for IR in HCV patients and this phenomenon is manifested even in the early stages of the disease [52].

Nevertheless, most studies found a correlation between IR /type 2 DM and the degree of liver disease. Our early observation that HCV patients who developed type 2 DM had higher grade of inflammation and fibrosis, compared with nondiabetic HCV patients [31] was later explained by several studies which showed that inflammation and fibrosis are significantly related to insulin resistance (Table 4). In an early small study, a significant negative correlation was found between insulin sensitivity and both fibrosis and histological activity index [54]. A similar correlation between insulin levels (a marker for IR) and fibrosis was found in overweight, but not lean, HCV patients [65]. In an elegant large study of 260 subjects with HCV and different stages of fibrosis, insulin sensitivity was evaluated by the HOMA-IR [52]. 121 patients had only stage 0 or 1 of hepatic fibrosis, and this sub-group had already significant higher level of insulin C-peptide and HOMA-IR compared with 137 healthy volunteers. Predictors of HOMA-IR in the whole group were: body-mass index, previous treatment, viral genotype and portal and periportal inflammation. Notably, although IR was evident even in subjects without fibrosis or only with minimal degree, IR increased with the progression of fibrosis [52]. In another large study of patients with various liver diseases, high insulin levels were found only in HCV patients, and in this group a gradual increase in fasting insulin levels with increasing fibrosis was noted [66]. Notably, insulin levels were only high in patients with detectable serum levels of HCV core. In a study of 56 non-diabetic and non-cirrhotic patients, HOMA-IR and insulin levels increased in parallel with the progression of fibrosis [67]. Interestingly, in patients with all degrees of fibrosis, HOMA-IR correlated with tumor necrosis factor (TNF-α) levels. Two other recent studies, confirmed the correlation between HOMA-IR and fibrosis [68,69], while in another study, a significant correlation between HOMA-IR and fibrosis was found in genotype 1 HCV patients in a univariate but not in a multivariate analysis [57].

A topic which has gained a lot of interest in recent years is liver steatosis. The overall prevalence of steatosis in HCV is about 50% and it is even more prevalent in subjects with HCV genotype 3 [71]. A recent study showed that in patients with genotype 1, steatosis correlated with HOMA-IR while in patients with genotype 3, steatosis correlated with viral load [57].

The sequence of events is still debated: Is HCV-induced IR the primary event leading subsequently to fibrosis? Another possibility raised is that steatosis is the primary event leading both to fibrosis and to insulin resistance. There are several data supporting the notion that IR is the primary event in non-3 genotype. Firstly, the findings that IR was evident even in HCV subjects without fibrosis or only with minimal degree [52]. Secondly, insulin sensitivity increased significantly after 4 months of interferon therapy in responders [49]. Thirdly, in a model of transgenic mice that specifically expressed the HCV core protein in the liver, the animals developed IR as early as 1 month old while hepatic statosis developed only after 3 months [72]. Fourthly, in genotype 3 despite extensive hepatic steatosis, there is a low incidence of IR [71]. All of these suggest that HCV infection leads to insulin resistance as a primary event in non-3 genotype HCV. Compensatory hyperinsulinemia that occurs in IR can lead to fibrogenesis. In hepatic stellate cells, incubation with insulin led to increased connective tissue growth factor mRNA, a key factor in the progression of fibrosis [73].

Table 4. The association between insulin resistance and liver inflammation and fibrosis in HCV.

Author, year, reference	Number of patients	Main findings
Konrad et al, 2000, [54]	10	Significant correlation between insulin sensitivity and histological activity index and fibrosis
Hickman et al. 2003, [66]	160	In overweight patients, insulin levels were independently associated with fibrosis.
Hui et al., 2003 [52]	260	Portal inflammation was an independent predictor of HOMA-IR. HOMA-IR was an independent predictor of fibrosis.
Kawaguchi et al., 2004, [67]	158	Increased fasting insulin and HOMA-IR were associated with the severity of hepatic fibrosis.
Maeno et al., 2003, [68]	56	HOMA-IR increased in parallel with the progression of fibrosis.
Muzzi et al., 2005, [69]	221	HOMA-IR was an independent predictor of fibrosis.
D'Souza et al. 2005, [70]	59	HOMA-IR was an independent predictor of fibrosis.
Fartoux et al. 2005, [57]	141	High insulin levels were predictor for fibrosis in a univariate analysis but not an independent predictor in a multivariate analysis

The development of steatosis in HCV patients can be related to insulin resistance. Insulin resistance is known to have a pivotal role in liver-fat accumulation and in the development of nonalcoholic fatty liver disease (NASH) [74]. Insulin induces the transcription of sterol regulatory element binding protein 1c (SREBP-1c), a key regulator of fatty acid synthesis in the liver. Overexpression of SREBP in mouse adipose tissue leads to fatty infiltration [75]. However, in HCV other direct mechanisms leading to steatosis have been described, mainly in genotype-3. In patients with HCV genotype 3, extensive steatosis occurs at an early stage of the disease in the majority of patients, correlating with viral load [71,76]. Several mechanisms by which HCV alters lipid metabolism have been identified including: inhibition of microsomal triglyceride transfer protein, oxidative stress, hyper-homocysteinaemia, and induction of genes such as stearoyl coenzyme A desaturase 4 [71,76]. Steatosis has a central role in the progression of fibrosis and it was found to be an independent predictor of fibrosis [57,71]. Lipid accumulation in the liver even without peripheral lipid accumulation, can in turn lead to hepatic insulin resistance [77] and reducing liver triglyceride content reversed hepatic IR [78]. Therefore a vicious circle is suggested: In non-3 genotype HCV infection leads to insulin resistance, leading to fibrosis and steatosis and the latter further augments IR and fibrosis. In genotype 3, viral proteins lead primarily to steatosis and subsequently to fibrosis.

MECHANISMS FOR INSULIN RESISTANCE IN HCV

In a study of nonobese/nondiabetic subjects with HCV compared with non-HCV patients, liver tissue was examined following incubation with insulin [79]. In liver tissue of HCV patients, but not in non-HCV patients, several defects were found in the insulin signal: decreased tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1), decreased IRS-1/p85 phosphatidylinositol 3-kinase (PI3-kinase) association and PI3-kinase activation and marked reduction in insulin stimulated Akt phosphorylation [79].

The impairment in insulin signaling can be related to increased levels of proinflammatory cytokines such as TNF-α, that occurs in HCV [80]. TNF-α producing cells, mainly of the macrophage /Kupfer lineage, are increased in HCV infection and activation of TNF-α showed significant correlation with the inflammatory process [62,63]. TNF-α has been shown by many studies to link obesity and IR [81,82]. Long-term exposure of animals to TNF-α induced insulin resistance, whereas neutralization of TNF-α increased insulin sensitivity [83]. TNF-α interferes with the insulin signaling pathway, particularly by inhibiting tyrosine phosphorylation of the insulin receptor and IRS proteins [84]. Emerging data suggest that a TNF- α inhibitory effect on insulin signaling is mediated by activating serine /threonine (Ser/Thr) kinases that phosphorylate the IRS proteins and uncouple them from their upstream and downstream effectors [85]. Inhibition of IRS proteins requires stimulation of c-Jun NH_2 - terminal kinase (JNK) and inhibitor kB kinase β (IKK β). Inhibition of IKK β prevents Ser/Thr phosphorylation of IRS proteins induced by TNF- α as well as by high-fat diet. TNF-α regulates expression of several adipocyte genes known to modulate insulin sensitivity [85,86]. These intriguing data link inflammatory process caused by various environmental stress-stimuli including chronic HCV infection, and major metabolic pathways [87]. The mechanisms for TNF-α induced insulin resistance are summarized in Table 5.

Table 5. Mechanisms for TNF-α induced insulin resistance.

- TNF-α inhibits insulin-stimulated phosphorylation of insulin receptor and IRS proteins by activating serine/threonine kinases that phosphorylate the IRS proteins and uncouple them from their upstream and downstream effectors.
- TNF-α down-regulates genes in adipocytes encoding proteins such as: adiponectin, PPAR-γ, GLUT-4
- TNF-α stimulates lipolysis, increasing free fatty acids and subsequently leading to insulin resistance in muscle and liver
- TNF-α has a direct inhibitory effect on insulin action in the liver
- TNF-α induces hepatic SOCS-3 expression subsequently leading to IR

Abbreviations:
TNF, tumor necrosis factor; PPAR, peroxisome proliferator-activated receptors;
GLUT, glucose transporter; SOCS, suppressors of cytokine signaling, IR, insulin resistance

Our own results support the hypothesis that TNF-α can link HCV infection and the development of type 2 DM [55]. Soluble TNF receptors (sTNFR) 1 and 2, considered to be reliable indicators of TNF-activation were measured in non-cirrhotic HCV patients with and without diabetes, type 2 DM patients, and healthy controls. Marked and significant increase

of both sTNFR1 and sTNFR2 were demonstrated in HCV patients with DM compared with the other 3 groups [55]. These results demonstrate that excessive activation of TNF-α characterizes HCV patients who develop DM and suggest that *TNF-α can play a central role in the pathogenesis of insulin resistance that leads to type 2 DM*. Further support for the role of TNF-α in IR is provided by a mouse model that specifically expressed the HCV core protein in the liver [75]. These animals developed IR at an early age, and glucose intolerance on a high-fat diet caused by a failure of insulin to suppress hepatic glucose production. The role of TNF-α in the pathogenesis of these abnormalities was strongly suggested by findings of more than 2-fold increase of TNF-α in the liver and by restoration of insulin sensitivity by TNF-α antibody [75]. Interestingly, high pretreatment intrahepatic TNF-α mRNA level is also a predictor of failure to respond to interferon therapy [88].

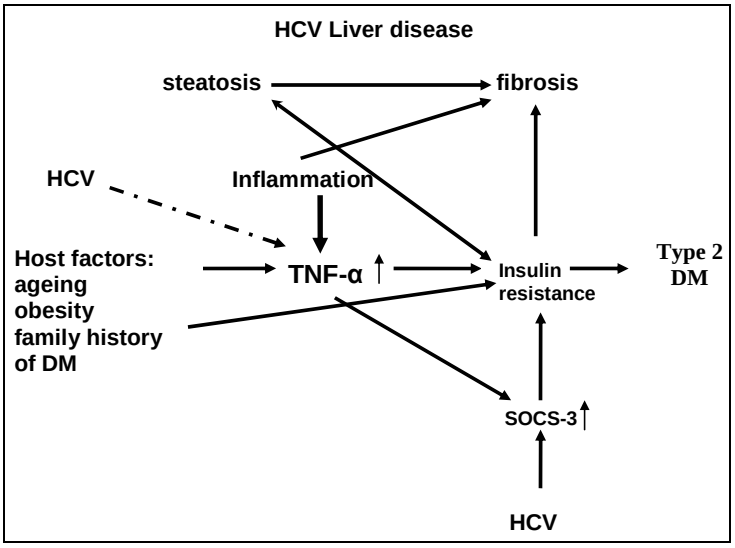

Figure: Proposed scheme of events in HCV infection (non-genotype 3). HCV-mediated liver-inflammatory process and possible direct effect of HCV, cause activation of TNF-α subsequently leading to insulin resistance (IR) in susceptible persons. Host factors such as ageing, obesity and family history of type 2 DM can augment IR either by increasing TNF-α levels or by other non-TNF independent mechanisms. TNF-α and HCV core protein induce hepatic expression of SOCS-3 also leading to IR. A bi-directional relationship between IR and steatosis exists and both IR and increased steatosis lead to progression of fibrosis. *Abbreviations: TNF, tumor necrosis factor; SOCS, suppressor of cytokine signaling.*

Proinflammatory cytokines that increase with HCV infection and HCV core protein, can up-regulate suppressor cytokine signaling (SOCS)-3, known to inhibit insulin signaling [67]. In human hepatoma cells, HCV core up-regulated SOCS-3 and caused ubiquitination of IRS-1 and IRS-2. These defects were not seen SOCS3-/- mouse embryonic fibroblasts cells or when an inhibitor of proteosomal proteolysis was added [67]. Recent data have shown that over-expression of SOCS-1 and SOCS-3 in obese animals led to the development of IR and hepatic steatosis and inhibiting the expression of SOCS proteins improved insulin sensitivity and hepatic steatosis [89]. The inhibitory effect of SOCS proteins on insulin signaling can be mediated by attenuating the activity of signal transducer and activator of transcription 3

(STAT-3), and mice lacking liver STAT-3 revealed IR. Interestingly injection of TNF-α into a mouse model, induced marked expression of SOCS-3 [90]. A recent study found a strong correlation between SOCS-3 and TNF-α mRNA in livers of HCV patients [91].

We suggest the following scheme in non-genotype 3 HCV infection (Figure). HCV-induced liver inflammation and possible direct effect of HCV, cause activation of TNF-α and subsequently, by the various mechanisms described above, to insulin resistance. Host factors such as ageing, obesity, family history of type 2 DM can augment insulin resistance either by increasing TNF-α levels or by other non-TNF independent mechanisms. TNF-α, and HCV core protein induce hepatic expression of SOCS-3 also leading to IR. A bi-directional relationship between IR and steatosis exists and both IR and increased steatosis lead to progression of fibrosis.

IMPLICATIONS AND APPROACHES TO TREATMENT

Assuming that about 180 million people worldwide are infected with HCV [2], that 144 million of those have chronic hepatitis and that about 36 million (~20%) have cirrhosis – than millions may be affected by the so called HCV – Diabetes association. At a conservative estimate, one third of the HCV-induced cirrhosis patients (12 million) and one fifth of those with chronic hepatitis (~28 million) have diabetes. Thus, 40 million people may have diabetes that is strongly associated with an infectious cause (HCV). This constitutes a major public health problem which may markedly increase if HCV-infected patients who have insulin resistance that falls short of diabetes (impaired fasting glucose, impaired glucose tolerance) – are also considered. Before reviewing the implications of concurrent HCV-Diabetes, these huge numbers suggest that *screening* for glucose abnormalities should be initiated in anti-HCV-positive patients [34]. An early detection might possibly allow for improved follow-up and better control. This may be no less important for the HCV-infected non diabetic subjects. In one small study, oral glucose tolerance test exposed 9/50 (18%) hitherto unrecognized diabetes patients [34] and in another, 7/71 hepatitis C patients who were free of diabetes (10%) became diabetic during a 7-year follow-up [65].

Since barely a decade has elapsed since the HCV-Diabetes link had been first recognized and much less, since insights into the mechanisms have been gained – both the current understanding of its implications and treatment considerations remain largely speculative and only partially understood. Nevertheless, several assumptions seem reasonably valid:

First, like other patients with diabetes, patients whose diabetes is associated with chronic hepatitis C are likely to be prone to the microvascular and macrovascular complications of diabetes. In fact one retrospective study even suggests that the course of microvascular disease in HCV patients may be worse than that of controls: patients with diabetic glomerulosclerosis that were comparable on renal biopsy, showed a significantly sharper decline of renal function when they had concurrent HCV infection than did similar patients who did not have chronic hepatitis C [92]. Also, during a follow-up period of just over two years, one third of the HCV patients required hemodialysis vs. 18% of the HCV-negative group (P=0.1). Thus, primary prevention measures with lifestyle modification, aspirin, tight blood pressure (and glycemic) control and possibly also a cautious use of statins are probably

indicated. This may be particularly true since type 2 diabetes as well as atherosclerosis are regarded today as having a significant inflammatory component and both occur more often and exhibit a worse course when markers of inflammation are increased [93-97]. TNF levels in particular, have been associated with carotid atherosclerosis [98] and with recurrent vascular events after myocardial infarction [99]. As a chronic inflammatory condition associated with increased levels of TNF in the liver and in the serum, hepatitis C - Diabetes may well be associated with more adverse vascular outcomes than either condition alone.

Second, as previously discussed (Figure), the literature suggests a vicious cycle in that more extensive liver inflammation and fibrosis may lead to higher glucose and hyperinsulinemia in susceptible persons, while the latter in turn, promote progression to fibrosis [52], that may further deteriorate glucose tolerance [80,100]. The initiating events in this vicious cycle remain hard to determine. However, a recent elegant study from Paris shows that at least in genotype 1 patients, insulin resistance is the cause rather than the consequence of steatosis and fibrosis. Moreover, hyperinsulinemia and associated steatosis ≥10% constitute prominent risk factors for extensive fibrosis [57]. The postulated central role of HCV-induced cytokines, primarily TNF-α, in the pathogenesis of insulin resistance remain an attractive hypothesis [55].

Third, one hitherto unconfirmed study from Japan suggests that increasing insulin resistance in patients with chronic hepatitis C may be a harbinger of increased extra hepatic manifestations [101].

Fourth, diabetes mellitus in HCV may increase the risk of these patients to develop hepatoma (HCC). A case-control study of primary liver cancers among US veterans revealed that diabetes alone was not associated with a significantly increased risk. However, when diabetes was associated with a chronic viral hepatitis such as HCV, the risk of hepatoma was significantly increased (adjusted odds ratios 1.57) [102]. When 279 patients with chronic hepatitis C in whom cirrhosis was excluded, were followed, HCC developed in 13 patients over a mean follow-up period of about 7 years. Only diabetes mellitus and age were associated with hepatoma in multivariate analysis [103]. Synergism between HCV and diabetes in hepatocarcinogenesis [104,105] must be carefully evaluated in future studies.

Fifth, diabetes mellitus in HCV-induced cirrhosis may be associated with poor survival [106]. The status of the glycemic control was identified as an independent predictor of survival (P=0.0018). In contrast, it had no predictive value in patients with HBV.

Sixth, there is enough evidence to support that liver damage is additive and possibly synergistic when more than one noxious stimuli are present. Therefore, close attention and attempt to correct any potentially reversible coexisting condition that may adversely affect the liver is important. This commonly includes obesity-associated nonalcoholic fatty liver disease (NAFLD) [74], alcohol consumption, iron overload in certain patients [107], etc.

In addition to screening HCV patients for glucose abnormalities and taking preventive measures common to all patients with diabetes, *two questions of central importance remain,* that are unique to HCV-Diabetes patients:

1. What is the role of current antiviral treatments in improving glucose tolerance and ameliorating diabetes?

2. Can treatment aimed at better control of the diabetes (such as with insulin sensitizing agents) improve the course of the chronic liver disease?

These questions are complex and can only be answered partially at present.

Antiviral therapy with interferon leading to clearance of HCV resulted also in restoration of insulin sensitivity [50]. However sustained response is attained in less than half of HCV patients and further modalities of therapy are needed [5]. As discussed before, insulin resistance that has a central role in the pathogenesis of the HCV-DM association, also adversely affects the course of the liver disease. Can we then improve chronic HCV liver disease by using measures to improve insulin sensitivity? Some data suggest that weight loss in HCV patients is associated with reduction of liver enzymes, steatosis and fibrosis [108]. Insulin sensitizing agents such as metformin and thiazolidinediones, have been shown in small studies to have a beneficial effect in NASH and may have also a role in HCV liver disease [109]. TNF-α inhibition that was shown in the transgenic animal model to restore insulin sensitivity, is another intriguing possibility [72]. TNF-α inhibition has been used successfully in rheumatoid arthritis and in inflammatory bowel diseases [110]. Initial and partial observations suggest that administration of anti-TNF antibodies to patients with chronic hepatitis C, does not adversely affect the chronic viral infection. Further large studies evaluating different treatment modalities of improving insulin sensitivity are needed to establish their role in chronic hepatitis C virus infection.

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Chapter V

HEREDITARY HEMOCHROMATOSIS

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ABSTRACT

Recent developments in the field of genetics and molecular biology have transformed the way we look at iron-related disorders, particularly hemochromatosis. This chapter presents a unifying concept of this disorder that is based on this new knowledge and stems from the idea that, beyond their genetic diversities, all known hemochromatoses originate from the same metabolic error, the genetic disruption of human tendency for circulatory iron constancy. Hepcidin, the iron hormone, holds a central pathogenic place in hemochromatosis, similar to insulin in diabetes: genetically determined lack of hepcidin synthesis or activity causes unrestricted release of iron from macrophages and intestine leading to tissue iron overload and disease.

In the past decade, the number of proteins implicated in iron homeostasis has increased dramatically; many of these have been characterized, their functions and regulatory pathways dissected; and genetic causes have apparently been identified for the major disorders associated with tissue iron overload. These dramatic steps forward have transformed the way we look at iron-related disorders, particularly hemochromatosis (HC) or hereditary hemochromatosis. The term "hemochromatosis" was coined in 1989 by Von Recklinghausen

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[1] to describe the necroscopic finding of massive organ damage associated with dark tissue staining caused by what he believed to be a blood-borne pigment. It was Sheldon, however, in his monumental 1935 review of all cases published in the world's medical literature [2], who suggested that the disorder was probably hereditary. For much of the 20th century, hemochromatosis was believed to be a monogenic disease [3-7]. In 1996, Feder et al. [8] discovered a pathogenic mutation (C282Y) involving a novel MHC class I-like gene, which was present in the majority of hemochromatosis patients throughout the world. However, as genetic testing for HFE mutations became more widespread, it rapidly became clear that the situation was more complicated than previously thought. In fact, we have seen the discovery of other iron genes whose mutations were associated with hereditary iron overload syndromes with some, or many, or apparently even all of the phenotypic features of classic hemochromatosis: transferrin receptor 2 (*TfR2*) [9], hepcidin (*HAMP*) [10], hemojuvelin (*HJV*) [11] and ferroportin (*FPN*) [12,13]. Is the hemochromatosis label valid for these syndromes as well? Over the past century, the definition of HC and classification of this iron-overload disorder has been changing, evolving, stretching, and twisting to accommodate an increasingly rapid and rich succession of the new discoveries, in particular, those of the genetics era. This review presents a concept of HC, based on this new knowledge, which stems from the idea that, beyond their genetic diversities, all known hemochromatoses belong to the same clinicopathologic entity as they all originate from the same pathophysiologic event [14].

I) Definition and Classification

Hemochromatosis is an iron loading disorder caused by a genetically determined failure to prevent unneeded iron from entering the circulatory pool and characterized by progressive parenchymal iron overload with potential for multi organ damage and disease. This definition includes the classic disorder related to HFE C282Y homozygosity (the prototype for this syndrome and by far the most common form) and the rare disorders more recently attributed to loss of TfR2, HAMP, or HJV. There exist four basic features that defines this disease (Table 1): hereditary nature (usually autosomal recessive); early and progressive expansion of the plasma iron compartment (increasing transferrin saturation); progressive parenchymal iron deposits with potential for severe damage and disease that may involve, liver, endocrine glands, heart and joints; non-impaired erythropoiesis and optimal response to therapeutic phlebotomy. If hemochromatosis is defined by the presence of all four of the features discussed above, other iron-overload syndromes can be excluded from this subset if they lack at least one of its defining characteristics (Table 2).

Table 1. Distinguishing features of hemochromatosis.

• Hereditary (usually autosomal recessive) trait
• Early and progressive increase of circulatory iron (i.e. high transferrin saturation) that precedes iron accumulation in tissues (i.e. high serum ferritin)
• Early and preferential iron deposition in parenchymal cells with potential for damage and diseases such as liver cirrhosis, cardiomiopathy, endocrinopathy, arthropathy
• Unimpaired erythropoiesis and optimal response to phlebotomy

Table 2. Human iron overload disorders.

HEREDITARY	ACQUIRED	MISCELLANEOUS
• Hereditary hemochromatosis (*HFE-, TfR2-, HJ-V, HAMP-related*)	• Dietary • Parental • Long-term haemodialysis • Chronic liver disease o Hepatitis C and B o Alcoholic cirrhosis, o NASH	• African siderosis [d] • Neonatal haemochromatosis [e]
• Ferroportin disease	• Porphyria cutanea tarda	
• Aceruloplasminemia [a]	• Post portacaval shunting	
• Atransferrinemia [b]	• Dysmetabolic iron overload syndrome	
• H-ferritin related iron overload [c]		
• Hereditary iron-loading anaemias		

[a] Ceruloplasmin is important in the release of iron from cells. Affected individuals present with progressive extrapyramidal signs, cerebellar ataxia, dementia, diabetes mellitus and hypochromic microcytic anemia [87,88]. [b] Iron transport and delivery to the bone marrow is impaired. The main clinical feature is severe anemia, while tissue iron overload results from a compensatory increase in intestinal iron absorption [90]. [c] Due to mutation in the regulatory region of H ferritin [91], but this single observation awaits validation by additional reports. [d] Particularly frequent among Africans who drink a traditional beer brewed in non-galvanized steel drums, the disorder was once exclusively attributed to dietary excess, segregation analysis has led to the conclusion that an unidentified iron-loading gene may confer susceptibility to the disease [92,93] while one modifier gene could be ferroportin [94]. [e] Massive hepatic iron loading and generally fatal perinatal liver failure whose hereditary nature is uncertain, although familial cases have been described [95].

II) MOLECULAR PATHOGENESIS

A. The Hemochromatosis Proteins

a) 1. HFE

HFE is a major histocompatibility class-I-like protein whose ancestral peptide-binding groove is too narrow to allow classic antigen presentation [15] while a possible non-classic activity has been recently proposed [16]. It is incapable of binding iron [17], while interaction between HFE and the transferrin receptor, TfR1, which mediates transferrin-bound iron uptake by most cells [17,18], has been fully documented although its biological effects are

still uncertain. At present, it is unclear whether the interaction of HFE with TfR1 is key for the pathogenesis of HC [19,20,21].

The C282Y mutation (substitution of tyrosine for cysteine at position 282 due to a single-base transistion, 845G->A), the most common pathogenic mutation of HFE, is associated with disruption of a disulfide bond in HFE that is critical for its binding to β2-microglobulin [22]. The latter interaction is necessary for the stabilization, [intracytoplasmic] transport and expression of HFE on the cell surface and endosomal membranes where HFE interacts with TfR1. The H63D mutation, a common HFE mutation whose pathogenic significance is still uncertain, does not impair HFE-TfR1 interaction. While the biological function of HFE is still unknown, circumstantial evidence indicate that it might be required for the synthesis of hepcidin, the iron hormone secreted by the hepatocytes (see below) (Figure 1).

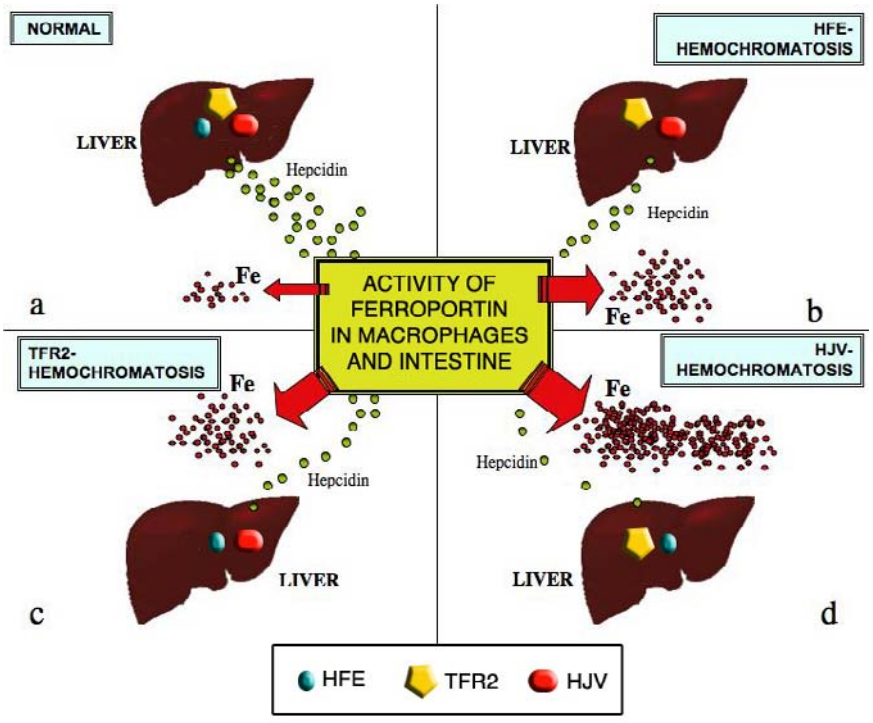

Figure 1. Hepcidin as a common pathogenic denominator in hemochromatosis. (A) In normal subjects circulatory iron sets a basal level of hepcidin synthesis by hepatocytes. Serum hepcidin modulates the amount of iron released from macrophages and enterocytes that contributes the pool of circulatory iron able, in a regulatory feed-back loop, to control the hepatic production of hepcidin. HFE, TfR2 and HJV are likely required for hepcidin activation in response to the circulatory iron signal (B) If HFE is non functional (i.e. HFE-related hereditary hemochromatosis) hepcidin synthesis by the hepatocytes is unregulated and inappropriately low, although a residual hepcidin activity will be still possible due to the presence of functional TfR2 and HJV: the consequent unrestricted release of iron from macrophages and enterocytes leads to progressive expansion of the plasma iron pool followed by tissue iron overload and organ damage. Circumstantial evidence indicates that also TfR2 may be required for iron sensing by the hepatocyte. Therefore, a similar pathogenic pathway may be shared by TfR2-related hemochromatosis (C). HJV is likely a more important regulator of hepcidin than HFE and TfR2. Therefore, a mutated HJV will lead to a more profound inhibitory effect on hepcidin synthesis, a more dramatic increase in circulatory iron and a more severe iron overload syndrome (D).

b) 2. Transferrin Receptor 2 (TFR2)

The gene for a second human transferrin receptor (*TfR2*) [23], unlike TfR1, is highly expressed in the liver and it is not regulated by intracellular iron status [24]. TfR2 mediates the uptake of transferrin-bound iron by hepatocytes [23], but its in vitro affinity for transferrin is 25–30-fold lower than that of TfR1 [25]. The biologic role and function of TFR2 remain unknown, but recent studies suggest a role for TfR2 in hepcidin synthesis in the liver. In fact, its putative role in hepatocyte uptake of iron [23] is difficult to reconcile with the hemochromatosis phenotype observed in humans with pathogenic TfR2 mutations [9] and in TfR2-knock-out mice [26]. Yet, its persistent hepatic expression during iron overload might conceivably reflect a contribution to the modulation of hepcidin synthesis in this setting (see below) (Figure 1).

c) 3. Hemojuvelin (HJV)

Hemojuvelin has been recently discovered while searching for the gene responsible for "juvenile" HC [11]. The putative full-length protein is 426 amino acids; it contains a C-terminal GPI-anchor, suggesting that it can be present in either a soluble or a cell-associated form. The function of hemojuvelin is presently unknown. However hepcidin levels are depressed in individuals with *HJV* mutations, [11] and in HJV knock-out mice [27]. In a recent study cellular hemojuvelin positively regulated hepcidin mRNA expression, and recombinant soluble hemojuvelin suppressed hepcidin mRNA expression in primary human hepatocytes in a log-linear dose-dependent manner, suggesting that HJV is a transcriptional regulator of hepcidin [28] (Figure 1).

d) 4. Hepcidin (HAMP)

Hepcidin, the long waited iron hormone, is an antimicrobial defensin-like peptide [29-31]. It is the product of the *HAMP* gene, constituted of 3 exons and 2 introns located on chromosome 7 and 19 in mouse and humans, respectively. Humans and rats have a single HAMP gene [31], whereas two functional genes, *Hamp 1 and 2* are present in the mouse genome [32]. Expression of hepcidin mRNA is nearly confined to the liver. The transcript encodes a precursor protein of 84 amino acids, including a putative 24-aa leader peptide while the circulating forms consist of only the C-terminal portion (20- and 25 amino acid peptides) [33].

Evidence from transgenic mouse models indicates that hepcidin is the principal down-regulator of the transport of iron across the small intestine and the placenta, and its release from macrophages. Transgenic animals over-expressing hepcidin die perinatally due to severe iron-deficiency anemia occurring in the context of reticuloendothelial cell iron overload [32]. In vivo injection of hepcidin into mice significantly reduced mucosal iron uptake and transfer to the carcass, independently on iron status or presence of HFE [34], or induces hypoferremia in humans [35]. The present view is that hepcidin down-regulates iron efflux from intestine and macrophages by interacting with the main iron export protein in mammals, ferroportin (FPN). In fact, it has been recently shown, that hepcidin binds to FPN in cultured cells stably expressing FPN, and, following complex internalization, leads to FPN degradation [36]. Moreover, hepcidin is highly concentrated in organs expressing FPN [35]. This implies decreased FPN expression, and reduced iron egress from cells such as

enterocytes and macrophages, whenever circulating hepcidin levels are high, namely, inflammation [31,37] and iron overload [31,38-40].

The stimulation of hepcidin during inflammation is indirect and appears to be mainly mediated by the inflammatory cytokine IL-6 [40-42], likely produced by Kupffer cells [43], whereas it is controversial whether HFE is involved in this activity [42-44]. Due to its sensitivity to inflammatory stimuli and owing to its effect on iron egress from macrophages and enterocytes, hepcidin is likely responsible, along with its cellular counterpart ferroportin, for iron trapping in enterocytes and macrophage during chronic inflammatory disorders, an iron disturbance eventually leading to "anemia of inflammation" or "anemia of chronic disease" [45].

As to the regulatory role of iron on hepcidin synthesis, it might be that serum iron or transferrin saturation is the signals for hepcidin up-regulation but the details of this stimulation are still obscure. In fact, exposure of cultured murine and human hepatocytes to iron salts [31] or iron-saturated transferrin [40] does not increase hepcidin mRNA and may even reduce it. At variance with their role in inflammation, Kupffer cells do not seem to be required for hepcidin stimulation during iron overload [43,46].

The fact that mice with genetic disruption of the transcription factors Upstream Stimulatory Factor 2 (*USF2*) or *C-EBPa*, both required for hepcidin transcriptional control, have an hemochromatotic phenotype [47,48] and human lacking hepcidin have a severe form of HC [10] places now hepcidin at the center of the pathogenesis of HC (see below) (Figure 1).

B) The Metabolic Abnormality in all Forms of HC

The first biochemical manifestation of hemochromatosis is an increase in the transferrin saturation, which reflects an uncontrolled influx of iron into the bloodstream from enterocytes and macrophages. Duodenal transfer of iron to the plasma is inappropriately high for body iron stores [49]. As a result, their intestinal iron absorption generally exceeds iron loss by approximately 3 mg / day [50]. The enhanced absorption of dietary iron by duodenal enterocytes plays an essential role in elevating total body iron, but macrophages are normally the source of most of the iron found in the plasma compartment [51]. In hemochromatosis, these cells seem to release more iron than their normal counterparts, and consequently they are invariably iron-poor [14]. The release of iron from both duodenal cells and macrophages, which is mediated by the iron exporter ferroportin (*FPN*), is normally down-regulated by the hepatic iron-regulating hormone, hepcidin. Indeed, the iron-overload syndromes associated with *HFE*, *TfR2*, *HAMP*, and *HJV* mutations are all characterized by inadequate hepcidin synthesis [11,39,52,53]. Its expression in the liver is also significantly impaired in HFE, TfR2 and HJV knock-out mice [27,54,55] and hepatic deposition of iron in HFE-KO animals can be prevented by hepcidin overexpression [56]. These findings suggests a unifying pathogenic model for all forms of HC in which HFE, TfR2 and HJV are all independent but complimentary regulators of hepcidin synthesis in the liver (Figure 1). When all three proteins function correctly (and the HAMP gene that encodes hepcidin is normal), the amount of iron transferred into the blood will be appropriate to body needs, and excessive

iron deposition in tissues will be avoided. The relative contributions of the three genes to this modulatory process may be different, with a more substantial role assigned to HJV based on the more severe iron overload phenotype associated with HJV mutations. Loss of *one* of the minor regulatory proteins (HFE- *or* TfR2-related HC) will result in an appreciable increase in iron influx into the bloodstream, but residual hepcidin activity will be sustained by the second minor regulator and the major regulator, HJV gene. The result is a mild “adult” hemochromatosis phenotype, with gradual plasma iron loading and gradual accumulation of iron in tissues. Loss of the "major" hepcidin regulator, HJV will produced a more dramatic effect on influx of iron into the bloodstream (not unlike the one produced by loss of hepcidin itself) and result in a more severe, “juvenile”, HC. Combined loss of HFE *and* TfR2 (*HFE+TfR2*-related HH) would theoretically result in much more rapid and substantial increases in plasma iron, and, consequently, greater iron overload in tissues, in short, a severe “juvenile” phenotype, as recently reported [53]. Finally, the complete loss of hepcidin (*HAMP*-related HH), in spite of normal HFE, TfR2, and HJV, will inevitably lead to massive uncontrolled release of iron into the circulation.

III) EPIDEMIOLOGY

HFE-related hemochromatosis is the most common form of HC and also the most frequently inherited metabolic disorder found in whites, with a prevalence of the pathogenic mutation ten times higher than that of cystic fibrosis. The C282Y mutation likely arose in a single individual, in this case a Celtic or Viking ancestor inhabiting northwestern Europe some 2000 years ago. The genetic defect, which caused no serious obstacle to reproduction and may even have conferred some advantages, was passed on and spread through population migration [57].

Whiel organ disease is highly unlikely in simple C282Y heterozygotes, 1%-2% of compound C282Y / H63D heterozygotes seem to be predisposed to expression of the disease [57]. The clinical significance of other seemingly rarer forms of compound heterozygosity, e.g., monoallelic C282Y or H63D mutation with substitution of cysteine for serine at amino-acid position 65 (S65C) or other rare changes on the second allele, is still being debated [14].

The frequency of TfR2 mutations is low and so far they have been detected in a few pedigrees throughout the world. *TFR2* gene is relatively large, spanning 21 kilobases and including 18 exons, thus, detection of new TFR2 mutations in single patients remains cumbersome. Analysis of TfR2 mutations should be especially considered in individuals with adult non-HFE hemochromatosis, particularly from families with high consanguinity.

Most cases of juvenile HC are due to mutations of *HJV* located on chromosome 2 [11]. To date 23 mutations have been identified in 43 juvenile HC families. One common mutation, G320V, has been reported in all studies. It is present in half of juvenile HC families. A small proportion of patients with the juvenile form of HC carry mutations in the gene encoding the iron regulatory peptide hepcidin on chromosome 19q13 [10].

IV) CLINICAL ASPECTS

A) Classic HFE HC

HFE-related hemochromatosis is a multifactorial disease characterized by step-wise progression from biochemical abnormality to organ toxicity [14]. The altered HFE protein plays an essential role in this process but its presence alone is insufficient to explain the broad spectrum of metabolic and pathologic consequences ascribed to the disease. Expressivity of the genetic defect may lead to biochemical abnormalities, symptoms and signs or overt organ disease. Early diagnosis in hemochromatosis is especially important since treatment by venesection before irreversible end-organ damage has occurred can restore a normal life expectancy [58-60].

Hemochromatosis should be suspected in a middle-aged men presenting with cirrhosis of the liver, bronze skin, diabetes and other endocrine failure, or joint inflammation and heart disease. However, this classical syndromic presentation is rare. Today diagnosis is made at earlier stages as an effect of screening and enhanced case detection due to greater clinician awareness and higher index of suspicion. The most common presenting symptoms are now fatigue, malaise, and arthralgia, while hepatomegaly is one of the earliest physical signs. Elevated serum transferrin saturation iron, which precedes increased serum ferritin, and moderately increased transaminase levels are common biochemical abnormalities. Increasing serum ferritin levels herald iron accumulation in tissues, and values above 1000 ng/ml may indicate underlying liver fibrosis in HFE-HC, even when transaminase levels are normal [61]. Once the diagnosis of HFE-HC is established, all family members, particularly siblings, should be subjected to a thorough biochemical and clinical evaluation, and genetic testing is advisable for adult first-degree relatives. Further details on HFE-HC are available elsewhere [62,63].

As specified, while all patients with overt HFE-related HC (i.e., with organ damage) carry the C282Y mutation on both HFE alleles, some C282Y homozygotes present no evidence of organ disease or biochemical abnormalities although they should still be considered to be at increased risk. It is currently impossible to predict whether (and to what extent) a C282Y homozygote will express the disease phenotype. At present, we can only conclude that, while the majority of C282Y homozygotes have laboratory evidence of plasma and tissue iron overload (i.e., high transferrin saturation and ferritin levels, respectively), organ disease requiring medical treatment is today much less common [64-68].

Although clinical descriptions of TfR2-related HC are currently limited, patients with TfR2 mutations almost invariably present signs of significant hepatic iron overload and express a systemic iron loading syndrome almost indistinguishable from that of HFE hemochromatosis [9,69-72].

B) “Juvenile” HC

The rather vague term, “juvenile hemochromatosis,” has been used to refer to a form of hereditary iron overload with a development pattern resembling that of adult HC but more

rapidly progressive. Because of the higher rate of iron loading associated with this disorder (and possibly differential tissue sensitivities to this massive toxic insult), cardiomyopathy and endocrinopathy, including reduced glucose tolerance, appear earlier than they do in adult HC, and death before the age of 30 is not uncommon [73,74]. We now know that this syndrome is usually associated with HJV or, in rarer cases, HAMP mutations (Table 3). The commonest symptom at presentation is hypogonadism, which, at the end of the second decade, may be present in all cases. In sporadic cases, also abdominal pain and cardiac disease represent common findings, while liver cirrhosis is recognized at later stages although silent micronodular cirrhosis is part of the syndrome.

Increased risk of clinically expressed disease has already been documented in patients with heterozygous mutations of both *HFE* and HAMP [75]. Reports of uncharacteristically severe disease in patients who apparently have TfR2 mutations alone, or in combination with HFE variants, might also be accounted for by undetected mutations of other hereditary hemochromatosis genes. The variety of genotypes that can produce a hereditary hemochromatosis phenotype highlights the importance of defining and classifying this disease as a unique clinicopathologic entity.

Therapeutic phlebotomy is the safest, most effective and most economical approach to treatment of all forms of HC. It can normalize life expectancy if initiated before organ damage has occurred. One unit (400-500 ml) of blood (containing approximately 200-250 mg of iron) is removed weekly until serum ferritin is less than 20-50 µg/L and transferrin saturation drops below 30%. Maintenance therapy, which typically involves removal of 2-4 units a year, can then be initiated and it must be continued for the duration of the patient's life to keep transferrin saturation and ferritin normal. Phlebotomy has little effect if started after organ impairment has already developed: the hypogonadism, cirrhosis, destructive arthritis, and insulin-dependent diabetes associated with HC are usually irreversible. Only if phlebotomy is contraindicated or non tolerated, other iron removal strategies (e.g use of deferoxamine or other iron chelators) should be considered.

V) THE FERROPORTIN DISEASE

The ferroportin disease (FD) (Table 2 and 3) is an hereditary iron storage disease distinct from HC. It is an autosomal dominant inherited disorder of iron metabolism which causes progressive iron retention predominantly in reticuloendothelial cells of the spleen and liver and is characterized by steadily increase of serum ferritin, inappropriately high as compared to the extent of serum transferrin saturation, marginal anemia, and mild organ disease [76].

The disorder was described clinically in 1999 [77] and associated with the A77D mutation of ferroportin (FPN) in 2001 [12,13]. The disorder has been now reported in many countries and, at variance with the distribution of the HFE gene mutations that appear to be restricted to Caucasians of northern European ancestry, it appears to be spread worldwide in different ethnic groups [76] (Table 3).

Table 3. Hereditary iron overload disorders in humans.

DISORDER	AFFECTED GENE (symbol / location)	KNOWN OR POSTULATED GENE PRODUCT FUNCTION [a]	GENETICS	MECHANISM FOR CELLULAR IRON ACCUMULATION	CLINICAL ONSET (decade)	MAIN CLINICAL MANIFESTATION
I. HEMOCHROMATOSIS	Hemochromatosis gene (HFE / 6p21.3)	• Interaction with transferrin receptor 1 • Hepcidin regulator				
	Transferrin-receptor 2 (TfR2 / 7q22)	• Uptake of iron-bound transferrin • Hepcidin regulator	Autosomal recessive	Increased iron influx	3°-5°	Liver Disease
	Hepcidin antimicrobial peptide (HAMP /19q13.1)	Down-regulation of iron efflux from macrophages, enterocytes, placenta			2°-3°	Hypogonadism and cardiac disease
	Hemojuvelin (HJV/ 1p21)	Hepcidin regulator				
II. Ferroportin Disease	Solute carrier family 40 (iron-regulated transporter), member 1 (SLC40A1 / 2q32)	Iron export from cells including macrophages, intestine, placenta	Autosomal dominant	Decreased iron efflux	4°-5°	Liver abnormalities Marginal anemia
III. Aceruloplasminemia	Ceruloplasmin (CP / 3q23-q25)	Iron efflux from cells	Autosomal recessive	Decreased iron efflux	2°-3°	Neurologic manifestations Anemia
IV. A(hypo)transferrinemia	Transferrin (Tf / 3q21)	Iron transport in the bloodstream	Autosomal recessive	Increased iron influx	1°-2°	Anemia

FPN is the main iron export protein in mammals. It is expressed in several cell types that play critical roles in mammalian iron metabolism, including placental syncytiotrophoblasts, duodenal enterocytes, hepatocytes and reticuloendothelial macrophages [78-80]. In vitro, as mentioned earlier, FPN has been found to be the cellular receptor for hepcidin [36] (Figure 1). A current pathogenic model for the FD is that loss-of-function mutations of FPN cause a mild but significant impairment of iron recycling particularly by reticuloendothelial macrophages [12], which normally must process and release a large quantity of iron derived from the lysis of senescent erythrocytes. As a consequence, iron retention by macrophages would lead to tissue iron accumulation (i.e. high serum ferritin) but decreased availability of iron for circulating transferrin (i.e. low transferrin saturation) and for bone marrow. At later stages, both iron retention in cells and activation of feedback mechanisms to increase intestinal absorption might contribute to more pronounced iron overload. Although the patients are not anemic in the adulthood, indicating that adequate iron is available for normal erythropoiesis, they may show a reduced tolerance to phlebotomy and become anemic on therapy in spite of persistently elevated serum ferritin values [12,77] (Table 3). It is possible that different mutations along the protein may differently affect the function of FPN and indirectly lead to variability in clinical expressivity. In this context, anecdotal evidence suggests that mutation of this gene can also be associated with parenchymal iron overload that closely resembles that of *HFE*-related hemochromatosis [81]. In addition, recent in vitro studies suggest that a subgroup of ferroportin mutations might lead to hepcidin "resistance" and increased rather than diminished iron export [82-84]. Therefore, a subgroup of patients with FD may carry gain-of-function mutations that lead to enhanced iron release from enterocytes and macrophages and a phenotype similar to classic HC. This hypothesis cannot be ruled out a priori, but it awaits validation by additional experimental data and more extensive clinical studies.

Although phlebotomy is an effective therapeutic tool, in some individuals a weekly phlebotomy program is not tolerated and slight anemia and low transferrin saturation are rapidly reached despite a still elevated serum ferritin level. With a less aggressive phlebotomy regimen, they can also be iron depleted, although a therapeutic target of serum ferritin <30 ng/ml, adopted for classical hemochromatosis, should be avoided due to the risk of anemia. Adjuvant therapy with erythropoietin may be beneficial. Discontinuation of phlebotomy treatment is followed by a rapid rise of serum ferritin.

The FD should be suspected in all cases of familial hyperferritinemia or in sporadic cases in the absence of known secondary causes (such as infection, dysmetabolism, inflammation and malignancy). Differential diagnosis should also consider the rare form of familial hyperferritinemia-congenital cataract syndrome, which is not associated with tissue iron overload [85,86], aceruloplasminemia [87,88], and dysmetabolic hepatosiderosis [89], present in dyslipidemic individuals.

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Chapter VI

IRON IN CHRONIC LIVER DISEASE

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ABSTRACT

Primary and secondary iron overload syndromes may result in chronic liver injury, ultimately leading to hepatic fibrosis and cirrhosis. Iron toxicity is mediated by a number of mechanisms including oxidative stress, with iron-catalyzed production of reactive oxygen species causing oxidative damage to lipids, proteins, and nucleic acids. Iron can also have pro-fibrogenic effects on the liver which are mediated via inflammatory cells, hepatic stellate cells and pro-inflammatory cytokines. Elevated iron stores have been observed in a range of liver disorders such as alcoholic liver disease (ALD), nonalcoholic steatohepatitis (NASH), chronic hepatitis C virus infection (HCV), and porphyria cutanea tarda (PCT). The C282Y mutation in HFE is over-represented in subjects with PCT suggesting a role for this mutation in the pathogenesis of iron loading in this disorder. However, no clear role for this mutation has been demonstrated in other liver disorders. A number of novel iron transport genes may be involved in the pathogenesis of iron loading in ALD, NASH, HCV and PCT. Iron reduction therapy has been shown to be beneficial in PCT but not in HCV, NASH or ALD.

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Keywords: Alcohol; Cirrhosis; End-stage liver disease; *HFE* gene; Hepatitis; Iron; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Porphyria cutanea tarda.

1. INTRODUCTION

It has long been known that serum and hepatic iron parameters can be increased in chronic liver diseases of diverse etiologies excluding classical primary and secondary iron overload disorders [1]. Hepatic iron deposition is commonly observed in cirrhosis irrespective of causation although its clinical significance is often unclear. While excess iron may be toxic, evidence continues to mount that lesser degrees of hepatic iron loading may worsen liver injury or hepatic fibrosis in non-hemochromatotic liver diseases. Iron deposition has been associated with more severe fibrosis in alcoholic liver disease, nonalcoholic steatohepatitis and viral hepatitis but not biliary causes of liver disease [2,3,4,5]. More importantly, iron deposition is associated with more advanced degrees of liver dysfunction, as demonstrated by the significantly higher Child-Pugh and MELD scores. It also occurs in well-compensated cirrhosis, where the presence of stainable iron on liver biopsy may be predictive of more rapid deterioration in liver function and progression to death or transplantation compared with patients without siderosis [6].

Assessment of iron status in chronic liver diseases other than classical primary and secondary iron overload syndromes is complex. Serum transferrin saturation and ferritin levels, while useful for the assessment of iron overload in conditions such as hereditary hemochromatosis, are not as useful in the determination of iron status in chronic inflammatory liver diseases due to the effects of inflammation and pro-inflammatory mediators on serum iron levels and hepatic transferrin and ferritin synthesis [7]. The hepatic iron concentration (HIC) measured from biopsy specimens has long been considered the gold standard for defining hepatic iron content [8]. The HIC can be determined from fresh or paraffin embedded tissue using colorimetric methods or atomic absorption spectrophotometry [9,10,11]. Semi-quantitative grading of iron deposition and cellular distribution can be also be accomplished using histological assessment of sections stained for iron using Perls' Prussian blue method [12]. More recently, the refinement of magnetic resonance imaging and measurement of R_2 relaxation rate has led to the availability of a non-invasive and rapid measurement of HIC which is more accurate than liver biopsy for assessment of liver iron stores [13,14,15].

2. IRON AND ALCOHOLIC LIVER DISEASE

Patients with alcoholic liver disease commonly have elevations of serum ferritin levels and transferrin saturation [16,17]. Increased levels of non-transferrin-bound iron (NTBI), a form of iron thought to be especially reactive, have also been described in active alcohol abusers and in alcohol-induced cirrhosis [18]. Despite these elevations, hepatic iron concentrations in alcoholic liver disease are usually normal or only slightly increased. There are several reasons why hepatic iron overload may occur in patients with alcoholic liver

disease. Intestinal iron absorption may be increased due to increased iron uptake (as seen in African dietary iron overload) or up-regulation of intestinal metal transporters. Anemia due to hemolysis, hypersplenism, or ineffective erythropoiesis and hypoxemia due to interpulmonary shunts or ventilation/perfusion mismatch may increase intestinal iron absorption through suppression of hepatic hepcidin production. Hepcidin is a key regulator of iron absorption which is influenced by anemia, hypoxia and iron [19]. When hepcidin levels are decreased, iron absorption from the gastrointestinal tract and iron release from reticuloendothelial cells in the marrow are increased [20]. Hepatic iron uptake may be upregulated in the presence of chronic liver disease and elevated concentrations of NTBI. Finally, portosystemic shunts are associated with increased hepatic iron deposition [21,22,23].

A significant independent relationship between hepatic stainable iron and fibrosis has been described in a study of 268 alcohol-dependent patients from France [24]. Because cirrhosis develops in only 20%–30% of heavy drinkers of alcohol, factors other than alcohol must be involved in the pathogenesis. Homozygosity for the C282Y mutation in the *HFE* gene may act as a co-factor in the genesis of liver injury related to alcohol [25]. It is well known that excessive alcohol consumption and elevated hepatic iron stores in hereditary hemochromatosis interact synergistically to enhance the development of advanced hepatic fibrosis and cirrhosis [26].

Controversy surrounds the role of heterozygosity for HFE mutations (C282Y or H63D) in increasing the severity of alcoholic liver disease. Some studies have shown that the presence of the C282Y mutation was strongly associated not only with the presence of alcoholic liver disease but with the presence of more advanced degrees of fibrosis or cirrhosis [21,27]. However, a study of 257 patients with alcohol related liver disease from the north of England demonstrated no effect of HFE mutations on the severity of alcoholic liver disease [28]. Likewise, a population based study from Australia did not report an increased susceptibility to excessive alcohol consumption in subjects carrying the C282Y HFE mutation compared with findings for control subjects [29,30]. Results of studies in animal models of alcoholic liver disease provide further support for the concept that iron and alcohol can act synergistically. Several studies have clearly demonstrated the synergistic effect of diet-induced iron overload and alcohol in the production of increased oxidative stress in the liver and the development of liver injury including hepatic fibrosis or cirrhosis [31,32].

3. Iron and Nonalcoholic Fatty Liver Disease / Nonalcoholic Steatohepatitis

Nonalcoholic fatty liver disease (NAFLD) including nonalcoholic steatohepatitis (NASH) is the most prevalent disorder of the liver in the United States [33]. Hepatic steatosis detected by magnetic resonance spectroscopy is found in 31% of adults in the United States [34] and in 33% of potential live liver donors undergoing liver biopsy [35]. The factors that lead to progressive hepatocellular damage after triglyceride accumulation are not well elucidated. It appears that alteration of local and systemic factors (particularly insulin resistance) that control the balance between the influx or synthesis of hepatic lipids and their

export or oxidation leads to hepatic triglyceride accumulation [36]. The steatotic liver is then thought to be vulnerable to secondary insults, which lead to hepatocellular inflammation and fibrosis. A variety of factors have been implicated to produce a second “hit”, including hormones derived from adipose tissue (adipocytokines), oxidative stress and gut-derived bacterial endotoxin [37].

The association between hepatic iron accumulation and NAFLD/NASH continues to be examined. Several studies have reported that 22 to 62% of individuals with fatty liver disease and NASH have elevated hepatic iron stores [38,39]. Despite showing that serum ferritin levels are increased in 20%–50%, and elevated transferrin saturation (>55%) is present in 5%–10% of patients with NAFLD, increased ferritin levels are often markers of liver inflammation and injury rather than iron overload [40].

Studies from Australia and the United States have shown an increased prevalence of the C282Y and H63D mutations in HFE in subjects of northern European origin and who have NASH, with both homozygosity and heterozygosity being over-represented [41]. It has been suggested that the H63D mutation may contribute to the pathogenesis of NASH in men as this minor mutation was significantly more common in men with NASH than in women [16,42,43]. Some support for the role of iron in NAFLD/NASH was provided by a study in which iron-depletion therapy in patients with NAFLD, even with normal body iron stores, resulted in the near normalisation of serum alanine aminotransferase levels and marked improvements in insulin sensitivity [44]. Another study has shown that phlebotomy therapy improves insulin resistance in subjects with hepatic iron overload [45]. Overall, it is generally thought that iron burden and *HFE* mutations do not contribute significantly to hepatic fibrosis in the majority of patients with NAFLD [46-51].

4. Iron and Viral Hepatitis

Abnormal iron studies in patients with hepatitis B were first described by Blumberg and colleagues[52-54]. Other studies have shown that persistent hepatitis B virus infection is associated with iron overload [55,56]. Modest iron removal in patients with chronic hepatitis B by the use of deferoxamine (Desferal) was reported to improve the response rate to interferon therapy and to decrease serum ferritin and hepatic iron concentrations [57,58]. Interest in the role of iron in hepatitis C began in 1992 when DiBisceglie et al. found that up to 36% of patients with chronic hepatitis C had elevated serum iron parameters [59]. Similar observations have subsequently been reported by other groups [60,61].

While iron is an essential element for the survival of cells, excess amounts can result in tissue injury [62]. A key question is whether the iron directly contributes to liver injury or whether it is simply a reflection of hepatocellular damage. The concept that iron can act in a synergistic fashion with other hepatotoxins has been described previously. Iron has been shown to be a synergistic factor in the pathogenesis of alcohol and carbon tetrachloride induced liver diseases [63-65]. It is generally accepted that iron increases the formation of reactive oxygen intermediates which can result in lipid peroxidation and oxidative damage to proteins and nucleic acids. This can result in organelle dysfunction, fibrosis and eventually hepatocellular carcinoma. While these findings were initially based on iron overload studies,

lipid peroxidation products have been shown in the plasma and liver of patients with chronic hepatitis C [66-68]. Farinati et al. found HCV may have a direct cytopathic effect on hepatocytes through the occurrence of iron-dependent lipid peroxidation [67]. Patients with chronic hepatitis C had significantly greater lobular inflammation, steatosis, serum ferritin levels and transferrin saturation, tissue iron, glutathione and malondialdehyde levels compared with patients with other forms of chronic hepatitis not related to HCV infection. These results suggested that altered serum iron parameters and hepatic iron accumulation in chronic hepatitis C may be related to a specific effect of the virus on parenchymal or non-parenchymal cell function. In liver, the lipid peroxidation products are mainly observed in portal tract macrophages [68]. Lipid peroxidation products have been shown to stimulate collagen production in activated hepatic stellate cells and cultured human fibroblasts [69,70]. Alternatively, lipid peroxidation products may increase production of TGF-β or other profibrogenic substances by Kupffer cells which might then stimulate hepatic stellate cell activation [71,72]. Iron could also contribute to the increased risk of hepatocellular carcinoma in chronic hepatitis C through DNA damage from iron-induced adduct formation and chromosomal damage [73-75].

Much evidence has accumulated supporting an immunopathological mechanism underlying liver injury in chronic hepatitis C [76-78]. Iron has been shown to increase the formation of reactive oxygen intermediates which lead to lipid peroxidation and subsequent oxidative damage to proteins and nucleic acids [79]. Virus specific T cells are present in the liver tissue and peripheral blood of patients with HCV infection and are able to contribute to hepatocellular injury, but are not able to eliminate viral infection [80,81]. Iron has been shown to impair antigen-specific immune responses and generation of cytotoxic T-cells, decrease functional T-helper precursor cells, and enhance T-suppressor activity [82,83]. Natural killer cell activity has also been reported to be decreased in iron overload conditions [84-86]. Lymphocyte proliferation is inhibited by ferritin [87,88]. Ferritin molecules, particularly those rich in heavy (H) subunits, bind to activated T-cells [89] and H-ferritin receptors are expressed by T-cell lines [90,91]. These data suggest that iron could impair host lymphocyte-dependent clearance of HCV virus. Alpha interferon possesses multiple actions including direct antiviral effects and enzyme modulation [92]. The actions of interferon are not known to be dependent on intracellular iron although it is possible that iron might also interfere in some way with these actions resulting in a reduced antiviral activity.

It has been suggested that transferrin and nontransferrin-bound iron uptake pathways may be affected in necroinflammatory conditions [93]. As a result, non-responders might have increased iron uptake and hepatic iron deposition compared with non-responders. Increased hepatic iron deposition in hepatitis C may then result in increased oxidative stress in the liver, decreased glutathione levels and lipid peroxidation and formation of malondialdehyde adducts. The type of molecule where the iron is stored could modulate these effects. Ferritin and hemosiderin release iron to different degrees, a property that may influence the ability of iron to participate in biological reactions [94].

Iron is known to affect immune mediated clearance of HCV by sinusoidal Kupffer cells and has also been shown to decrease Kupffer cell production of pro-inflammatory cytokines [95,96]. Kupffer cells from iron loaded animals exhibit reduced proinflammatory cytokine production compared with Kupffer cells from control animals. Thus iron loading may impair

immune clearance mechanisms via impaired macrophage function or interfere with the actions of interferon alpha on macrophage function. This is supported by observations that iron deposition within zone 1, portal tracts and sinusoidal lining cells is associated with a higher likelihood of non-response to interferon therapy [97,98]. There are reports of impaired phagocytic function by monocytes in hereditary hemochromatosis [99,100]. and bactericidal activity of macrophages in iron overload [101]. Interleukin 2 production by cytotoxic T-cells is reduced in the presence of iron overload.

There has been much interest in the role of iron as a determinant of response to antiviral therapy of HCV. Interferon alpha forms the cornerstone of effective treatment for hepatitis C [102-105]. There are several characteristics which are known to affect outcome of interferon treatment, including age, gender, duration of infection, mode of acquisition, degree of fibrosis on histology, HCV genotype and viral load, and iron status [106-113]. Treatment efficacy is enhanced by combining therapy with ribavirin and may potentially be improved further by optimizing other factors which influence treatment response. Further improvements have been possible with the use of long-acting, pegylated interferon plus ribavirin, such that cures are now possible in up to 60% of patients [114,115].

Van Thiel et al. examined the HIC of patients with a variety of different chronic viral hepatitis pathologies and found that it was lower in the group of patients who responded to treatment than in those who were non-responders [116]. It has been suggested that an HIC of greater than 1100 micrograms/gram was predictive of non-response in nearly 90% of patients [117,118]. Following these reports, investigators began evaluating the possibility that patients might benefit by being depleted of iron by repeated therapeutic phlebotomy before treatment with interferon to improve response rates in previous non-responders.

Therapeutic phlebotomy alone has been shown to reduce serum aminotransferases in patients with hepatitis C [119]. In a study of 8 patients with chronic hepatitis C who had previously failed to respond to treatment with interferon alpha, serum ALT levels fell in 7 of 8 following iron reduction [120]. Hayashi et al. reported that iron reduction alone led to the normalization of serum ALT levels in 5 of 10 patients with chronic hepatitis C [121]. Four to 13 phlebotomies, with removal of 1-3 g of iron, over 2-9 months were required to achieve iron removal as judged by serum ferritin levels less than 10 ng/ml. Seven patients underwent repeat biopsy within 2 months of iron depletion, with no apparent change in the severity of portal fibrosis or inflammation. This was followed up in a long-term study of Japanese patients who had not experienced a complete or sustained virological response to interferon. Therapeutic phlebotomies were performed until a state of iron depletion was achieved, defined as a serum ferritin level of less than 10 ng/ml [122]. The iron depletion was then maintained by further phlebotomies. Mean serum levels of ALT decreased from 117 to 75 IU/l and remained at less than 72 IU/l for the ensuing 5 years. The severity of hepatic fibrosis in the group subjected to iron reduction decreased from 2.3 to 1.7 by the Desmet scoring system ($p<0.05$). In control subjects not subjected to phlebotomy, the mean value at baseline was 1.7 and the mean value at follow-up was 2.0 ($p>0.05$). The severity of inflammation increased in 1 of the 13 in the chronic-iron-reduction group, whereas it increased significantly in 12 of 13 control subjects.

Van Thiel et al. randomized 30 non-responders to iron depletion followed by interferon-α or interferon-α alone [123]. Twelve of 15 (80%) of patients treated with iron depletion and

interferon had a virological response at 6 months compared with 6/15 (40%) in the interferon-alone group. Significantly higher sustained virological response rates were seen in the iron depleted group (60%) compared with interferon-alone group (13%). Iron chelation with deferoxamine has also been shown to improve response to interferon therapy [124]. However, there have been no clear effects of iron reduction on levels of HCV RNA in serum [125,126].

Fong et al. conducted a randomized study that evaluated the effect of iron depletion on aminotransferase activity, HCV RNA levels and response to interferon alpha therapy in patients with chronic hepatitis C [127]. Serum ALT levels decreased in 15 of 17 patients after phlebotomy. Changes in iron indices and ALT levels were not accompanied by changes in HCV RNA levels. At the end of 24 weeks of interferon therapy, similar numbers of phlebotomized patients (7 of 17) had a response compared to control patients (6 of 21). However after 6 months of follow up, 5 of 17 phlebotomized patients remained HCV RNA negative compared with 1 of 21 controls ($p=0.07$). Tsai et al. have also shown that phlebotomy therapy may result in a sustained virologic response in up to 15% of patients who have previously not responded to treatment with interferon but who are retreated following phlebotomy therapy [128].

Boucher et al. found no difference in the HIC between responders and non-responders to treatment with interferon and noted that the HIC decreases with IFN treatment whether or not patients respond clinically [129]. However, they did identify a relationship between HIC and inflammatory activity such that the iron load was higher in those patients with the greatest degree of histological inflammatory activity. Interestingly, HIC decreased following treatment with interferon. This was related to iron depleted from sinusoidal cells and was apparent regardless of whether patients responded to interferon therapy. These findings suggest that increased iron stores may be present in patients with chronic hepatitis C predominantly as a result of the degree of inflammatory activity, presumably correlating with cell injury or necrosis, with subsequent phagocytosis by Kupffer cells resulting in progressive increases in Kupffer cell iron loading. Pianko et al. showed that non-responders to interferon monotherapy tended to have a higher HIC, and following combination therapy with ribavirin, the sustained virological response rate was not affected by the HIC [130]. Rulyak et al. also demonstrated that HIC is not an independent predictor of response to therapy with interferon and ribavirin and that the HIC is not changed following combination therapy, regardless of baseline histology or virologic response [131].

Two multicenter, prospective, randomized trials have examined iron reduction as an adjuvant therapy to interferon in previous non-responders and interferon-naïve patients. DiBisceglie et al. showed that patients in the phlebotomy and interferon group exhibited a significant improvement in histological necroinflammatory activity but no benefit in viral clearance [132]. Fontana et al. demonstrated that iron reduction improved liver histology but also reduced end of treatment HCV RNA levels [133]. Disappointingly, this did not correlate with any significant sustained viral eradication after 6 months. Similar negative results have been described by others [134,135]. Sievert et al. examined the response to treatment of a cohort of 28 adult patients with β thalassemia major, transfusion-acquired severe iron overload and chronic hepatitis C infection [136]. Following 6 months of interferon treatment, 8 patients (28%) achieved a virological and biochemical response which was sustained for a

mean of 66 months. Interestingly, the HIC was uniformly high in all patients and had no effect on the outcome of treatment. Factors which did predict poor response to treatment included high levels of HCV RNA and the presence of HCV genotype 1. Previous studies in children have shown response rates to interferon of up to 40% despite the presence of increased hepatic iron [137,138]. In both of these studies non-responders appeared to have higher hepatic iron content. The responders and non-responders had similar HCV RNA levels. There were no significant relationships between HCV RNA levels and the HIC, the presence of elevated serum ferritin levels, or the ALT level. Many additional studies have been published regarding the role of iron in chronic hepatitis C [139-147]. Most have confirmed that increased serum and/or hepatic iron parameters are associated with a lower likelihood of response to interferon therapy.

Banner et al. conducted a study of the frequency with which stainable iron occurred in the livers of patients with chronic hepatitis C [148]. These investigators noted that non-responders to treatment had greater accumulation of iron in the sinusoids and portal tracts. Ikura et al. found that the presence and degree of portal iron deposition correlated inversely with the response to interferon treatment [149]. The presence of stainable iron has been shown to correlate with inflammation and fibrosis in chronic hepatitis C, suggesting that the iron came from damaged hepatocytes [150,151]. In contrast, the absence of stainable iron is associated with a higher likelihood of response [152]. Other groups have suggested that iron may be a more significant factor in certain genotypes, in particular genotype 1b. In a study by D'Alba et al. patients with chronic hepatitis C and genotype 1b had higher hepatic iron concentrations compared with other genotypes [153]. Genotype and hepatic iron concentration remained predictive factors of non-responsiveness on multivariate analysis.

The discovery of the *HFE* gene containing two missense mutations which result in C282Y and H63D substitutions in the protein and are strongly associated with impaired iron metabolism raised the possibility that abnormal *HFE* genotypes could contribute to iron-related cell injury in chronic hepatitis C [154]. A number of studies have analysed the relationship of *HFE* mutations and iron overload in chronic hepatitis C [155-157]. Most studies indicate that chronic hepatitis C in combination with homozygosity for the C282Y mutation results in earlier and more significant liver injury disease than either condition alone [158-162]. In general, subjects with chronic hepatitis C have frequencies of *HFE* mutations that are no different from the general population and simple heterozygous status for C282Y or H63D is not known to be a risk factor for liver disease in HCV. The product of the *HFE* gene is a major histocompatibility complex-I–type protein, and several immunologic differences have been described in subjects with *HFE* mutations compared with findings for those without [163,164].

In summary, iron influences the response of chronic hepatitis C to monotherapy with interferon alpha but does not seem to be a major factor in combination antiviral therapy with ribavirin. The mechanisms responsible for the effects of iron are not clear but emerging data suggest that the cellular location of iron within the liver lobule and the subsequent effects on immune function are likely to be critical determinants for these effects.

5. IRON AND PORPHYRIA CUTANEA TARDA

Porphyria cutanea tarda is caused by a defect in the functioning of uroporphyrinogen decarboxylase (UROD). UROD catalyses the conversion of uroporphyrinogen to coproporphyrinogen in the biosynthesis of heme, and enzymatic dysfunction results in accumulation of uroporphyrins within the skin resulting in the dermatological sequelae. Sporadic (type I) PCT accounts for approximately 80% of cases, has normal gene expression but the specific hepatic enzymatic activity of UROD is reduced by 60% [165]. In familial (type II) PCT there are a variety of autosomal dominant inherited gene mutations that display a low penetrance [166]. There is some evidence for a putative type III PCT that appears to be a familial form of type I PCT [167,168]. Finally, there is a toxic form of PCT where exposure to aromatic hepatotoxic hydrocarbons results in a cutaneous eruption similar to that of sporadic PCT, which forms the basis of an animal experimental model for PCT [169].

Abnormal iron metabolism in PCT has been long observed, and in 1970 Lundvall clearly demonstrated significant iron storage in the livers of 30 patients with PCT [170,171]. Hepatic siderosis and steatosis are commonly observed in PCT, while cirrhosis is less common and is seen in around 10% of cases. There may be an increased risk of hepatocellular carcinoma in patients with PCT [172-174].

Hereditary hemochromatosis is a common disease of excess iron storage in target organs such as the liver, heart and pancreas [175]. In 1976 a strong association was established between hereditary hemochromatosis and HLA-A3 [176]. As the hepatic siderosis of PCT and hereditary hemochromatosis appeared similar, investigators screened PCT patients for the HLA allelic markers. It was postulated that there might be a common genetic abnormality that could explain the iron overload in PCT patients. Kushner et al. reported a single family pedigree that appeared to support a link with sporadic PCT and HLA-A3 [177]. Fifty-seven percent of their patients with sporadic PCT were HLA-A3 positive. Subsequent investigators both reaffirmed and contradicted this observation [178-182]. Thus the issue of a common gene defect in hereditary hemochromatosis and PCT remained unanswered.

The frequency of the C282Y and H63D mutations in patients with PCT was subsequently examined. Roberts et al. demonstrated that 44% of patients with PCT carried at least one C282Y mutation compared with 11% of controls [183]. They found no difference in the incidence of the H63D mutation between patients and controls. Santos et al. described a similar incidence of the C282Y mutation in fifteen PCT patients, but a 23% incidence of the H63D mutation in PCT patients compared with 4% of controls [184]. The prevalence of C282Y and H63D mutations in Australian patients with PCT was similar to that described by Roberts [155]. Italian patients with PCT, that had previously shown a strong HLA-A3 linkage in 1996, demonstrated no increased incidence of the C282Y mutation, but did show an increased incidence of the H63D mutation [185].

It is well described that phenotypic expression of PCT is aggravated by external agents such as alcohol, estrogens or HCV infection [186-188]. There are conflicting results relating to the prevalence of HCV infection in patients with PCT. Patients with PCT from Southern Europe have a high prevalence of antibodies to HCV, whereas PCT patients from Northern Europe have low prevalence of HCV antibody positivity [189]. Martinelli et al. showed 65.5% of the PCT patients in Brazil were positive for antibody to HCV [190]. This study also

reported a 17.4% incidence of the C282Y mutation in 23 patients with sporadic PCT compared with 4% in controls. Interestingly, they found no increased incidence of the H63D mutation which is more in keeping with the findings in groups studying patients of a Northern European ancestry.

How do HFE mutations or HCV infection influence the pathophysiology of sporadic PCT? It is likely that iron or HCV infection effect hepatocyte UROD activity. The importance of iron is clearly demonstrated by the beneficial effect that venesection has on the course of PCT. Furthermore, there is an increased incidence of PCT in South African populations which also have a high incidence of iron overload. It has been suggested that UROD inactivation is in part an iron-dependent process [191]. Neither ferrous nor ferric forms of iron have a direct effect on UROD. However, *in vitro* studies show that iron-dependent hydroxyl radical generating systems oxidize uroporphyrinogen into products that inhibit UROD. In toxic PCT, hydrocarbons may induce the activity of a cytochrome P450 family that oxidizes uroporphyrinogen; this process has been shown to be promoted by iron. It has also been postulated that iron induces the activity of ALA-synthetase which would promote the accumulation of uroporphyrins.

The exact function of HFE has yet to be determined, however, there is accumulating evidence to show that it does have a direct physiological role in iron absorption and thus when dysfunctional leads to the pathology seen in hereditary hemochromatosis [154]. Thus in susceptible individuals, hepatocytes may become iron loaded and UROD activity is inhibited.

The relationship of HCV infection to disturbances in iron metabolism is far more uncertain. Current emphasis has concentrated on the effect that iron has on the infected hepatocyte and hepatic immune function. It is accepted that iron-loaded patients with HCV infection have a less favorable outcome and are less responsive to anti-viral therapies [105]. What remains uncertain is whether the iron loading is a consequence of infection, or a host independent factor, that leads to a more severe outcome. Pro-inflammatory cytokines produced as a result of HCV infection could alter hepatic iron metabolism. The observation that Northern European PCT patients have a high prevalence of the C282Y mutation yet low HCV positivity with the converse observation in Southern European PCT patients, reinforces the suggestion that the final insult to UROD is an increase in intracellular iron.

6. IRON AND PORTOSYSTEMIC SHUNTING

Patients who have undergone portosystemic shunt often develop increased hepatic iron deposition [192,193]. Iron loading to the extent observed in typical hemochromatosis has been reported in a few patients who have undergone portosystemic shunt [194,195]. The reasons for hepatic iron accumulation after shunt placement are unknown. Early animal studies showed that duodenal iron absorption was increased following shunt procedures and that the increase can be reversed by duodenal exclusion, strongly supporting the suggestion that increased absorption of iron by the duodenum is responsible at least in part for increased hepatic iron loading [196]. In addition to increased duodenal absorption, other mechanisms have been postulated as causes of portosystemic shunt–related iron overload, including

relative hepatic hypoxia, and associated pancreatic insufficiency with decreased bicarbonate secretion [197].

7. IRON AND END-STAGE LIVER DISEASE

While increases in liver iron are relatively modest and infrequent in chronic hepatitis C virus patients, increased stainable iron and/or liver iron content are observed more commonly in livers with advanced fibrosis [3,4,198]. Together with the observed association of more severe fibrosis in alcoholic liver disease and NASH there is a strong suggestion that hepatic iron deposition seen in these conditions is neither a direct result of the specific underlying disease nor *HFE* mutations but rather related to advanced liver fibrosis. Serum iron indices and hepatic iron concentrations are often increased in patients with end-stage liver disease. Further, iron overload of a magnitude consistent with hereditary hemochromatosis has been reported in approximately 10% of cirrhotic livers removed at the time of liver transplantation [199,200]. Most patients with iron overload and end-stage liver disease do not have typical hereditary hemochromatosis, although an increased prevalence of heterozygosity for *HFE* mutations has been reported in some studies [201,202].

Kayali et al. demonstrated the association of siderosis with more advanced stages of cirrhosis with significantly higher Child-Pugh and MELD scores among siderotic patients [6]. Furthermore, the presence of siderosis was linked with a significant reduction in projected 5-year survival without liver transplantation even when the effect of Child-Pugh score on survival was taken into account. Despite being associated with more advanced degrees of liver dysfunction, the presence of iron deposition in well-compensated cirrhosis appears to be predictive of more rapid deterioration in liver function compared with patients without siderosis.

Hepatic iron overload may also be associated with decreased survival after liver transplantation in patients with *HFE*-associated hereditary hemochromatosis as well as in those without hereditary hemochromatosis. Using data from the National Hemochromatosis Transplant Registry, Kowdley et al. demonstrated that survival after liver transplantation among patients with iron overload are significantly lower than those without iron overload [203]. Crawford et al. published similar results showing reduced post-transplantation survival in hereditary hemochromatosis, with recurrent hepatocellular cancer as the most common cause of death [204]. The transplanted organs in hereditary hemochromatosis patients rarely reaccumulate iron, however in normal recipients of iron-loaded grafts late function may be compromised by slow mobilization of iron stores. Affected patients require careful clinical evaluation of perioperative and postoperative risk factors with iron depletion prior to liver transplantation possibly improving post-transplantation survival, particularly among patients with hereditary hemochromatosis.

8. CONCLUSIONS

The effect of hepatic iron as a co-factor in the pathogenesis of chronic liver disease has been evaluated in a variety of chronic liver diseases. Iron can have pro-fibrogenic effects on the liver which are mediated via inflammatory cells, hepatic stellate cells and pro-inflammatory cytokines. Elevated iron stores have been observed in a range of liver disorders such as ALD, NAFLD/NASH, HCV, and PCT.

The C282Y mutation in HFE is over-represented in subjects with PCT suggesting a role for this mutation in the pathogenesis of iron loading in this disorder. However, no clear role for this mutation has been demonstrated in other liver disorders. Iron influences the response of HCV to monotherapy with interferon alpha, but not to combination therapy with ribavirin. The cellular location of iron within the liver lobule and the effects on immune function are likely to be determinants for the mechanisms responsible for the effects of iron. Iron reduction therapy has been shown to be beneficial in PCT but not in HCV, NAFLD/NASH or ALD.

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Chapter VII

WILSON DISEASE

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DEFINITION

Wilson disease is an autosomal recessive inherited disorder of copper metabolism resulting in pathological accumulation of copper in many organs and tissues. The hallmarks of the disease are the presence of liver disease, neurologic symptoms and Kayser-Fleischer corneal rings.

The incidence of Wilson disease was estimated to be at least 1:30,000-50,000 with a gene frequency of 1:90 to 1:150. Among selected groups of patients Wilson disease is certainly more frequent. About 3 to 6% of patients transplanted for fulminant hepatic failure and 16% of young adults with chronic active hepatitis of unknown origin have Wilson disease.

PATHOGENESIS

The basic defect is the impaired biliary excretion of copper resulting in the accumulation of copper in various organs including the liver, the cornea and the brain. The consequence of copper accumulation is the development of severe hepatic and neurological disease. Copper's unique electron structure allows these cuproenzymes to catalyze redox reactions, but causes ionic copper to be very toxic, readily participating in reactions that promote the synthesis of damaging reactive oxygen species. Copper overload particularly affects mitochondrial respiration and causes a decrease in cytochrome C activity. Damage to mitochondria is an

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early pathological effect in the liver. Damage to the liver has been shown to result in increased lipid peroxidation and abnormal mitochondrial respiration both in copper loaded dogs and in patients with Wilson disease. The mechanism(s) triggering copper-induced lipid peroxidation are unknown.

The pathogenesis of neurologic disease is less clear. ATP7B is also expressed in the brain, but its function is unknown. It is conceivable that increased copper uptake into the brain is a direct result of certain mutation resulting in specific functional alterations of cerebral ATP7B. Neuronal damage is mediated by copper deposition in the brain [1]. Copper may be directly toxic to neurons or may exert its effects by selective inhibition of brain MAO-A. Copper accumulation in the brain may be secondary to liver damage, but this hypothesis is inconsistent with the clinical observation that many patients with neurologic disease have only mild liver disease, and that conversely patients with advanced liver failure have no neurologic symptoms. Furthermore the preferential affection of basal ganglia cannot be explained.

The Wilson Disease Gene

ATP7B is the gene product of the Wilson disease gene located on chromosome 13 and resides in hepatocytes in the *trans*-Golgi network [2,3]. The functionally important regions of the Wilson disease gene are six copper binding domains, a transduction domain (amino acid residues 837-864; containing a Thr-Gly-Glu motif) involved in the transduction of the energy of ATP hydrolysis to cation transport, a cation channel and a phosphorylation domain (amino acid residues 971-1035; containing the highly conserved Asp-Lys-Thr-Gly-Thr motif), an ATP-binding domain (amino acid residues 1240-1291) and eight hydrophobic transmembrane sequences (1–8), in one of which (region 6) is the cys-pro-cys sequence found in all P-type ATPases [4,5]. Alternatively spliced forms of WDP lacking transmembrane sequences 3 and 4 (exon 8) are expressed in brain.

Molecular genetic analysis of patients reveals over 200 distinct mutations (database maintained at the University of Alberta -http://www.medgen.med.ualberta.ca). Mutations include missense and nonsense mutations, deletions, and insertions. Some mutations are associated with a severe impairment of copper transport resulting in severe liver disease very early in life; other mutations appear to be less severe with disease appearance in mid adulthood. While most reported mutations occur in single families, a few are more common. The His1069Gln missense mutation occurs in 30 to 60% of patients of Eastern-, Northern- and Central-European origin. It is less frequent in patients of Mediterranean descent and only rarely seen in patients of non-European origin. The 2299insC mutation can be detected in some patients of European and Japanese descent. The Arg778Leu mutation is present in upto 60% of patients from Far-East. In Sardinia two frameshift mutations (1515insT and 2464delC) are found in about 20% of patients. These mutations were not found in other populations.

The study of genotype-phenotype correlations is hampered by the lack of clinical data, the rarity of some mutations, and the high frequency of the presence of two different mutations in individual patients (compound heterozygotes). In an ongoing study involving

820 pts with Wilson disease mostly from Europe, mutations on both chromosomes were identified in 58% of the patients, at least one mutation in 30%. Sufficient information is available only for the H1069Q mutation. Homozygosity for H1069Q is associated with late onset neurologic disease. In contrast, patients with mutations in exons 8 and 13 are commonly present with liver disease.

Hepatic Copper Metabolism and the Role of ATP7B

Copper is an essential nutrient needed for such diverse processes as mitochondrial respiration (cytochrome C), melanin biosynthesis (tyrosinase), dopamine metabolism (DOPA-ß-monooxygenase), iron homeostasis (ceruloplasmin), antioxidant defense (superoxyde dismutase), connective tissue formation (lysyl oxydase), and peptide amidation.

Dietary copper intake is approximately 1–2mg/day. Quoted copper contents of foods are unreliable. While some foods, such as meats and shellfish, have consistently high concentrations, others such as dairy produce are consistently low in copper. However, the copper content of cereals and fruits varies greatly with soil copper content and the method of food preparation. Estimates of copper intake should include water copper content, and the permitted upper copper concentration for drinking water is 2mg/L. Approximately 10% of dietary copper is absorbed in the upper intestine, transported in the blood loosely bound to albumin, certain amino acids and peptides. Finally, most of the ingested copper is taken up by the liver. Copper homeostasis is critically dependent on the liver because this organ provides the only physiologically relevant mechanism for excretion of this metal. Within the hepatic parenchyma, the uptake and storage of copper occurs in hepatocytes, which regulate the excretion of this metal into the bile. Copper appears in the bile as an unabsorbable complex, and as a result, there is no enterohepatic circulation of this metal.

The hepatic uptake of diet-derived copper occurs via the copper transporter 1 (Ctr1) which transports copper with high affinity in a metal-specific, saturable fashion at the hepatocyte plasma membrane [6,7]. After uptake by hepatocytes copper is bound to metallothionein (MT), a cytosolic, low molecular weight, cystein-rich, metal binding protein. MT I and MT II are ubiquitously expressed in all cell types including hepatocytes, and have a critical role to protect intracellular proteins from copper toxicity [8,9]. The copper stored in metallothionein can be donated to other proteins. Specific pathways allow the intracellular trafficking and compartmentalization of copper, ensuring adequate cuproprotein synthesis while avoiding cellular toxicity (Figure 1).

Metallochaperones (like ATOX 1) transfer copper to the site of synthesis of copper containing proteins [10,11]. The cytoplasmic copper chaperone ATOX1 is required for copper delivery to ATP7B by direct protein-protein interaction [12,13]. ATP7B is abundantly expressed in hepatocytes and is localized in these cells to the late secretory pathway, predominantly the *trans*-Golgi network. With increasing intracellular copper concentrations, this ATPase traffics to a cytoplasmic vesicular compartment that distributes near the cannalicular membrane in polarized hepatocytes and is critical for copper excretion [5,14]. Copper is incorporated into apoceruloplasmin at the level of the Golgi compartment [15]. Ceruloplasmin contains six tightly bound copper atoms. Its main function is to carry copper

to various tissues. Another important physiologic role of ceruloplasmin is to act as ferrooxidase, converting Fe^{++} to Fe^{+++}. Other chaperones (Sco1, Sco2, Cox17, lys7) carry copper for synthesis of the other cuproenzymes and do not require an interaction with ATP7B.

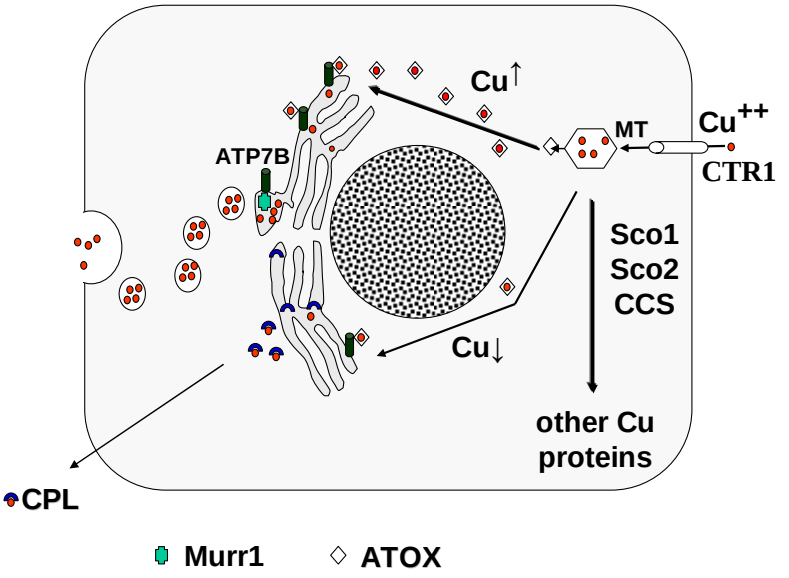

Figure1. Model of hepatobiliary copper transport. CTR1= copper transporter 1, MT= Metallothionein, CPL= ceruloplasmin, ATOX, Sco1, Sco2, CCS – copper chaperones.

Biliary excretion is the only mechanism for copper elimination, and the amount of copper excreted in the bile is directly proportional to the size of the hepatic copper pool.

Because hepatic uptake of dietary copper in not saturable, hepatic copper accumulation can easily be induced. Toxicity of copper, however, depends on its molecular association and subcellular localization rather than on its concentration in the liver. Metallothionein-bound copper is nontoxic. Several metals including zinc can induce metallothionein synthesis.

CLINICAL PRESENTATIONS

Wilson disease may present at any age, the oldest reported case was 76 years at the time of diagnosis. The clinical symptoms are highly variable, the most common ones being liver disease and neuropsychiatric disturbances. Children usually present with liver disease, while in older patients neurologic disease is more common. None of the clinical signs is typical and diagnostic. One of the most characteristic features of Wilson disease is that no two patients, even within a family, are ever quite alike. With increased awareness for Wilson disease patients are generally diagnosed earlier, thus “late“ consequences of the disease like Kayser-Fleischer rings or severe neurologic symptoms are less frequently seen. Early symptoms, if present at all, are uncharacteristic and nonspecific. Patients presenting with acute or chronic hepatic Wilson disease are indistinguishable from patients with liver diseases of other

etiology. Early neurologic symptoms are also quite untypical, and may progress slowly over many years before diagnosis is made based on “typical signs“. About half of the patients are referred for psychological testing because of poor school performance or behavioral problems.

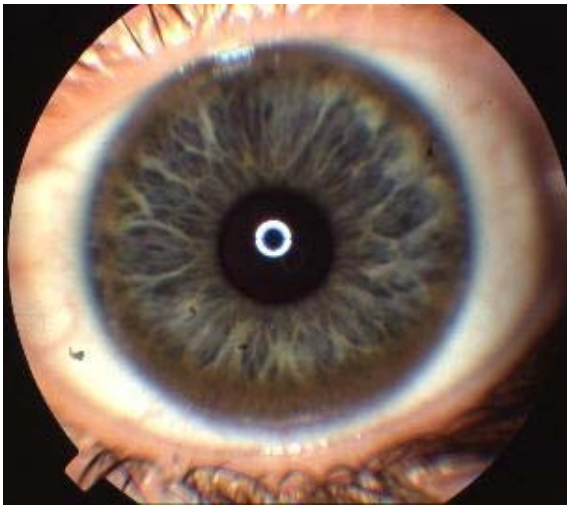

Figure 2. Kayser-Fleischer ring in a 15 year old patient with neurologic Wilson disease.

Kayser-Fleischer Rings

Characteristically, the ring starts as a small crescent of golden brown granular pigment seen at the top of the limbus. This is followed by the appearance of a lower crescent, and these two crescents gradually broaden, meet laterally and form complete rings (figure 2).The finding of a complete ring therefore suggests long-standing disease and is a useful indicator of severe copper overload. The ring is not always detected by clinical inspection. If doubt exists, the cornea should be examined under a slit lamp by experienced ophthalmologists. Kayser-Fleischer rings are present in 95% of patients with neurologic symptoms, in 50-60 % of patients without neurologic symptoms, and only in 10% of asymptomatic siblings.

Liver Disease

Most patients with Wilson disease, whatever their clinical presentation, have some degree of liver disease. Chronic liver disease (if undiagnosed and untreated) may precede manifestation of neurologic symptoms for more than ten years. Patients can present with liver disease at any age. The most common age of hepatic manifestation is between 8 and 18 years, but cirrhosis may already present in children below the age of 5. On the other hand, Wilson disease is diagnosed also in patients presenting with advanced chronic liver disease in their 50´-is or 60´-is, without neurologic symptoms and without Kayser-Fleischer rings.

Depending on referral patterns the proportion of patients presenting with liver disease alone varies from 20% to 46%. Liver disease may mimic any forms of common liver conditions, ranging from asymptomatic transaminasemia to acute hepatitis, fulminant hepatic failure (about 1 out of 6 patients with hepatic presentation), chronic hepatitis, and cirrhosis (about 1 out of 3 patients) with all of its complications.

Acute Wilsonian Hepatitis and Fulminant Wilson Disease

Acute wilsonian hepatitis is indistinguishable from other forms of acute (viral or toxic) liver diseases. It should be suspected in young patients with acute hepatitis non A-E. Liver histology often reveals the presence of cirrhosis. This initial episode of liver damage may be self-limiting and may resolve without treatment, and diagnosis is made retrospectively, when neurologic symptoms occur years later.

On the other hand the disease may rapidly deteriorate and resemble fulminant hepatic failure with massive jaundice, hypoalbuminemia, ascites, severe coagulation defects, hyperammonemia and hepatic encephalopathy. Hepatocellular necrosis results in the release of large amounts of stored copper. Hypercupriemia results in hemolysis and severe hemolytic anemia complicates acute liver disease. Although Wilson's disease is a rare disease, in patients presenting with fulminant hepatic failure it is not uncommon and accounts for 6 to 12% of patients with fulminant hepatic failure referred for emergency liver transplantation.

Although fulminant and subfulminant liver failure due to Wilson's disease has several distinctive features, rapid diagnosis may be very difficult. Serum aminotransferase activity is usually not increased above 10 times normal and much lower than the values commonly recorded in fulminant hepatitis. The combination of anemia, marked jaundice and relatively low aminotransferase activities in young patients should always raise the suspicion of acute Wilson's disease. The conventionally used parameters of copper metabolism are of little use. Kayser-Fleischer corneal rings and neurological abnormalities are absent in most patients presenting with acute liver disease. An alkaline phosphatase-total bilirubin ratio below 2.0 has been claimed to provide 100% sensitivity and specificity to diagnose Wilsonian fulminant liver failure, but the usefulness of this test was not confirmed in larger series. The best diagnostic test is the quantification of copper in biopsy material or in the explanted liver. One puzzling feature of fulminant Wilson disease is the preponderance of female sex (female: male ratio 3:1).

Chronic Hepatitis Due to Wilson Disease

Wilson disease may present, particularly in young patients, with a clinical syndrome indistinguishable from chronic active hepatitis of other etiology [16]. Symptoms include malaise, fatigue, anorexia, and vague abdominal complaints. Arthralgias, amenorrhea, delayed puberty, low grade jaundice may be present. Frequently, Kayser Fleischer rings are absent and plasma ceruloplasmin is in the normal range. Liver biopsy shows severe chronic active hepatitis but diagnosis is missed if hepatic copper content is not measured. Suspicion for Wilson disease should be high in young persons with chronic active hepatitis of unclear etiology. In this group Wilson disease is a common diagnosis. Without treatment, patients progressively deteriorate with ascites, edema and occasionally jaundice within few months, and eventually die of liver failure.

About half of patients presenting with neurologic symptoms may also suffer from significant liver disease. In a substantial proportion symptomatic liver disease predates the occurrence of neurologic signs.

Neurologic Presentation

Neurologic symptoms usually develop in mid-teenage or in the twenties. However, there are well documented cases in which neurologic symptoms developed much later (45-70 years). The initial symptoms may be very subtle abnormalities such as mild tremor, speech and writing problems and are frequently misdiagnosed as behavioral problems associated with puberty. The symptoms may remain constant or progress steadily. The hallmark of neurologic Wilson disease is a progressive movement disorder. The most common symptoms are dysarthria, dysphagia, apraxia, and a tremor-rigidity syndrome (“juvenile Parkinsonism“). Because of increasing difficulty in controlling movement, patients become bedridden and unable to care for themselves. Ultimately, the patient becomes helpless - usually alert, but unable to talk. In patients presenting with advanced liver disease, neurologic symptoms are mistaken as signs of hepatic encephalopathy.

Psychiatric Presentation

About one-third of patients initially present with psychiatric abnormalities. Symptoms can include reduced performance in school or at work, depression, very labile mood , sexual exhibitionism, and frank psychosis. Frequently, adolescents with problems in school or work are referred for psychological counseling and psychotherapy. Among our patients two were hospitalized in psychiatric institutions for psychosis, one having committed several suicide attempts and two for severe alcohol abuse before diagnosis of Wilson´s disease was made. The delay in diagnosis in one case was 12 years.

Other Clinical Manifestations

Hypercalciuria and nephrocalcinosis may be the presenting signs in patients with Wilson disease. Hypercalciuria is possibly the consequence of a tubular defect in calcium reabsorption. Penicillamine therapy was accompanied by a decrease in urinary calcium excretion to normal values in half of the patients studied. Wiebers et al. observed renal stones in 7 of 54 patients with Wilson disease.

Cardiac manifestations in Wilson's disease include arrhythmias, cardiomyopathy, cardiac death, and autonomic dysfunction. Thirty-four percents of patients with Wilson's disease have electrocardiographic abnormalities. Two cases of cardiac deaths were reported (one died of repeated ventricular fibrillation, the other, of dilated cardiomyopathy). In one of them copper content in the myocardium was measured and found to be markedly elevated.

The occurrence of *chondrocalcinosis and osteoarthritis* in Wilson disease may be due to copper accumulation similar to the arthropathy of hemochromatosis.

DIAGNOSIS

The diagnosis of Wilson disease is usually made on the basis of clinical findings and laboratory abnormalities (see table 1). According to Scheinberg and Sternlieb [17], diagnosis of Wilson disease can be made if two of the following symptoms are present: Kayser-Fleischer rings, typical neurologic symptoms and low serum ceruloplasmin levels.

Table 1. Routine tests for diagnosis of Wilson disease.

test	**typical finding**	**false "negative“**	**false "positive“**
serum ceruloplasmin	decreased	Normal levels in pts. with marked hepatic inflammation overestimation by immunologic assay	low levels in: - malabsorption - aceruloplasminemia - liver insufficiency - heterozygotes
24 hr urinary copper	>100 µg/d	normal: - incorrect collection - children without liver disease	increased: - hepatocellular necrosis - contamination
serum "free“ copper	>10 µg/dl	normal if ceruloplasmin overestimated by immunologic assay	
hepatic copper	>250 µg/g dry weight	due to regional variation - in pts with active liver disease - in pts with regenerative nodules	cholestatic syndromes
Kayser-Fleischer rings by slit lamp	present	- in up to 40% of patients with hepatic Wilson disease - in most asymptomatic siblings	primary biliary cirrhosis

Patients with Neurologic Disease

In a patient presenting with typical neurologic symptoms and having Kayser-Fleischer rings the diagnosis is straight forward. Clinical neurologic examination is more sensitive than any other method to detect neurologic abnormalities. No further diagnostic procedures are necessary to establish the diagnosis. Kayser Fleischer rings are rarely absent in neurologically symptomatic patients. However, there are a few well documented cases of neurologic Wilson

disease without demonstrable Kayser-Fleischer rings. In such patients diagnosis is usually made by a low serum ceruloplasmin level.

Brain magnetic resonance imaging (MRI) is useful to document the extent of changes in the central nervous system. The most common abnormalities are changes in signal intensity of gray and white matter, and atrophy of the caudate nucleus, brain stem, cerebral, and cerebellar hemispheres. A characteristic finding in Wilson disease is the “face of the giant panda“ sign, but is found only in a minority of patients. In Wilson disease, an abnormal striatum or an abnormal pontocerebellar tract correlates with pseudoparkinsonian-, and an abnormal dentatothalamic tract with cerebellar signs. On treatment some of the MRI abnormalities are fully reversible.

Auditory evoked brainstem potentials are helpful to document the degree of functional impairment and the improvement by decoppering treatment [18,19].

Patients with Liver Disease and Hemolytic Anemia

Diagnosis is far more complex in patients presenting with liver diseases. None of the commonly used parameters alone allows a certain diagnosis of Wilson disease. Usually a combination of various laboratory parameters is necessary to establish the diagnosis.

Kayser-Fleischer rings may be absent in up to 50 % of patients with Wilsonian liver disease and even in a higher proportion in fulminant Wilson disease. On the other hand patients with primary biliary cirrhosis may occasionally have Kayser-Fleischer rings.

Laboratory Parameters

Routine Laboratory Parameters of Liver Disease

In general, transaminases are only mildly increased, and deep jaundice combined with mild elevation of liver enzymes should raise the suspicion for fulminant Wilson disease. However, increases of transaminases may be indistinguishable from findings seen in acute hepatitis. Sometimes alkaline phosphatase activities are relative low in patients with Wilson disease. A ratio of total serum bilirubin concentration and alkaline phosphatase activity (>2) may differentiate fulminant Wilson disease from other forms of fulminant hepatic failure. However, the usefulness of this test was not confirmed in larger series.

Serum Ceruloplasmin

Serum ceruloplasmin can be measured by an immunologic assay or by the oxydase method. Since the immunologic ceruloplasmin assay can be automated by nephelometric methods, it is widely used in clinical laboratories. The oxydase method is only performed in specialized centers. Whereas serum ceruloplasmin is decreased in most patients with neurologic Wilson disease, it may be in the low normal range in up to 45% of patients with hepatic disease [20]. On the other hand, even a low ceruloplasmin level is not diagnostic for Wilson disease in the absence of Kayser Fleischer rings. It may be low in subjects with familial hypoceruloplasminemia, in celiac disease, in severely malnourished subjects, and in heterozygous carriers of the Wilson disease gene [21]. Thus, in patients with liver disease a

normal ceruloplasmin level cannot exclude, nor is a low level sufficient to make the diagnosis of Wilson disease. An overestimation of serum ceruloplasmin can be suspected if the serum copper concentration is lower than expected by the measured ceruloplasmin (which contains 0.3% of copper) level. Finally, ceruloplasmin is an acute phase reactant and its serum concentration increases as consequence of inflammation. Most patients with normal ceruloplasmin had marked liver disease. Similarly serum ceruloplasmin may increase in pregnancy to high normal values.

Serum Copper

In general, serum copper values parallel those of ceruloplasmin. Therefore, serum copper is frequently low in patients with Wilson disease. However, about half of patients have serum copper levels in the normal range. Patients with fulminant Wilson disease and/or hemolytic anemia may even have markedly increased levels. Most of the copper in serum is bound to ceruloplasmin, and under normal condition less than 5% circulates as “free copper“ and does not exceed 10 µg/dl in normal subjects. The “free“ copper concentration can be calculated by subtracting from the total copper concentration the ceruloplasmin bound copper (ceruloplasmin times 3.3).

Urinary Copper Excretion

Urine copper excretion is markedly increased in patients with Wilson disease; however, its usefulness in clinical practice is limited. The estimation of urinary copper excretion may be misleading due to incorrect collection of 24-hour urine volume or to copper contamination. In presymptomatic patients urinary copper excretion may be normal, but increase after D-penicillamine challenge [22]. On the other hand urinary copper excretion is also increased in any disease with extensive hepatocellular necrosis.

Hepatic Copper Content

Hepatic copper content exceeds 250 µg/g dry weight (normal: up to 50) is increased in 82% of patients with WD. In the absence of other tests suggestive for abnormal copper metabolism, diagnosis of Wilson disease cannot be made based on an increased hepatic copper content alone. Patients with chronic cholestatic diseases, neonates and young children and possibly also subjects with exogenous copper overload have increased hepatic copper concentration >250 µg/g. On the other hand, hepatic copper content may be normal or borderline in about 18 % of patients with unquestionable Wilson disease due to sampling given great regional differences in hepatic copper distribution, especially in the cirrhotic liver. Thus, estimates from a single biopsy specimen may be misleading.

Hepatic copper content was measured in 106 liver biopsies obtained at diagnosis of Wilson disease, in 212 patients with a variety of noncholestatic liver diseases, and 26 without evidence of liver disease [23]. Liver copper content was >250 µg/g dry weight in 87 (82%) patients, between 50 and 250 µg/g in 15, and in the normal range in 4. Liver copper content did not correlate with age, the grade of fibrosis, or the presence of stainable copper. Liver copper content was >250 or between 50 and 250 µg/g dry weight in 3 (1.4%) and 20 (9.1%) of 219 patients with noncholostatic liver diseases, respectively. By lowering the cut off from >250 to 75 µg/g dry weight the sensitivity of liver copper content to diagnose Wilson disease

increased from 81.2 to 96%, the negative predictive value from 88.2 to 97.1%, but the specificity (98.6 to 90.1%) and the positive predictive value (97.6 to 87.4%) decreased. Thus, although liver copper content is a useful parameter it neither proves nor excludes Wilson disease with certainty.

Diagnosis of Wilson disease requires a combination of a variety of clinical and biochemical tests. A diagnostic scoring system (table 2) was developed at the 8th International Meeting on Wilson disease, Leipzig/Germany [24] and its validity was confirmed by a retrospective analysis of a larger cohort of pediatric cases [25].

Table 2. Scoring system developed at the 8th International Meeting on Wilson disease, Leipzig 2001 [24].

Typical clinical symptoms and signs		**Other tests**	
KF rings		**Liver copper** (in absence of cholestasis)	
Present	2	>5xULN (>250µg/g)	2
Absent	0	50-250µg/g	1
Neurologic symptoms		Normal (<50µg/g)	-1
Severe	2	Rhodanine pos. granules*	1
Mild	1	**Urinary copper** (in absence of acute hepatitis)	
Absent	0		
Serum Caeruloplasmin		Normal	0
Normal(>0.2g/l)	0	1-2x ULN	1
0.1-0.2g/l	1	>2x ULN	2
<0.1g/l	2	Normal, but >5xULN after D-pen	2
Coombs' neg. hemolytic Anemia		**Mutation Analysis**	
		2 chromosome mutations	4
Present	1	1 chromosome mutation	1
Absent	0	No mutations detected	0

TOTAL SCORE	**Evaluation:**
4 or more	Diagnosis established
3	Diagnosis possible, more test needed
2 or less	Diagnosis very unlikely

* If no quantitative liver copper available

Liver Biopsy

Light Microscopy

Liver biopsy findings are generally nonspecific and not directly helpful to make the diagnosis of Wilson disease. Liver pathology includes early changes like fatty intracellular accumulations, which often proceed to marked steatosis. At later stages, hepatic inflammation with portal and periportal lymphocytic infiltrates, presence of necrosis and of fibrosis may be indistinguishable from other forms of hepatitis. Some patients have cirrhosis without any inflammation. The detection of focal copper stores by the Rhodanin stain is a pathognomic feature of Wilson disease but is only present in the minority (about 10%) of patients.

Electrone Microscopy

The ultrastructural abnormalities include pathological changes of mitochondria and peroxisomes. Hepatocellular mitochondria are pleomorphic, with varying combinations of abnormalities including enlargement, bizarre shapes, and increased matrix density, separation of the normally apposed inner and outer membranes, widened intercristal spaces, enlarged granules, and crystalline, vacuolated, or dense inclusions. Sometimes peroxisomes are abnormally enlarged, rounded, or misshapen, and contain a granular or flocculent matrix of varying electron density.

Radiocopper-Test

The basis of this test is the biphasic plasma kinetics of copper. Four hours following a tracer dose of ^{64}Cu, > 95% is removed from the circulation by the liver, and within 24 hours, 6% to 8% reappears incorporated into ceruloplasmin [26]. This second peak is absent in Wilson disease patients [27]. This test is rarely used today.

Mutation Analysis

Direct Mutation Analysis

Direct molecular-genetic diagnosis is difficult because of the occurrence of many mutations, each of which is rare [28]. Furthermore, most patients are compound heterozygotes (i.e. carry two different mutations). Direct mutation diagnosis is only helpful, if a mutation occurs with a reasonable frequency in the population. In Northern, Central and Eastern Europe [28] the most common mutations are: H1069Q mutation (allele frequency: 43.5%), mutations of exon 8 (6.8%), 3400delC (3%) and P969Q (1.6%). In other parts of the world the pattern of mutations is different (ie. Sardinia: UTR –441/-427del, 2463delC [29]; Far East: R778L [30,31]. Screening for mutations is done by denaturating HPLC analysis followed by direct sequencing of exon suspected to carry a mutation. This approach is impractical for clinical diagnosis. In contrast, using allele-specific probes; direct mutation diagnosis is rapid and clinically very helpful, if a mutation occurs with a reasonable frequency in the population (Table 3.) In Austria, the H1069Q mutation is present in 61% of Wilson disease patients, and a two-step PCR based test for this mutation became very useful. A multiplex PCR for the most frequent mutations makes direct mutation analysis for diagnosis feasible.

Haplotype Analysis

Because of the complexity in identifying the many mutations in Wilson disease, haplotypes can be used to screen for mutations and to examine asymptomatic siblings of index patients. A number of highly polymorphic microsatellite markers have been described that closely flank the gene and are highly variable: D13S316, D13S314, D13S301, D13S133 [32]. Where the markers are different at each locus in a patient, testing of at least one parent/or child of the patient is necessary to obtain the haplotype. The identification of unusual haplotypes can lend to support, but is not sufficient to confirm the diagnosis of Wilson disease.

Microsatellite markers are also useful to study the segregation of the Wilson disease gene in most families. By these approach diagnostic dilemmas in differentiating heterozygote gene carriers and affected asymptomatic siblings can be solved [33,34]. For such analysis, at least one first degree relative and the index patient is required.

Table 3. Common mutations of the WD gene in various populations.

Area (Ref)	Most common mutation (exon)	Other common mutations (exon)
Central-, Eastern-, Northwestern Europe [28,47]*	H1069Q (14)	3400delC (15), exon 8 (multiple), P969Q (13)
Sardinia [29]	-441/-421 del (5' UTR)	2463delC, V1146M
Canary Islands [48]	L708P (8)	
Spain [49]	M645R (6)	L1120X (15)
Turkey [29]**	P969Q, A1003T (13)	Exon 8, H1069Q,
Brasil [50]	3400delC (15)	
Saudi Arabia [51]	Q1399R (21)	
Far East [30,31]	R778L (8)	

* Russia, Bjelorus, Poland, Bulgaria, former Yugoslavia, Slovakia, Hungary, Germany, Benelux, Greece (Ferenci P, unpublished data); ** Ferenci P (unpublished data)

Family Screening

Once diagnosis of WD was made in an index patient evaluation of his family is mandatory. The likelihood to find a homozygote among siblings is 25%, among children 0.5%. Testing of second degree relatives is only useful if the gene was found in one of the immediate members of his/her family. No single test is able to identify affected siblings or heterozygote carriers of the WD gene with sufficient certainty. Today, mutation analysis is the only reliable tool for screening the family of an index case with known mutations; otherwise haplotype analysis can be used. A number of highly polymorphic microsatellite markers that closely flank the gene allow tracing the WD gene in a family.

TREATMENT

Treatments for Wilson disease progressed from the intramuscular administration of BAL to the more easily administered oral penicillamine. Alternative agents to penicillamine like trientine were developed and introduced for patients with adverse reactions to penicillamine. Zinc was developed separately, as was tetrathiomolybdate, which was used for copper poisoning in animals. Today, the mainstay of treatment for Wilson disease remains lifelong pharmacologic therapy, but the choice of the drug mostly depends on the opinion of the treating physician and is not based on comparative data. Based on the recent AASLD practice guideline on Wilson disease initial treatment for symptomatic patients should include a

chelating agent (penicillamine or trientine). Treatment of presymptomatic patients or maintenance therapy of successfully treated symptomatic patients can be accomplished with the chelating agent penicillamine or trientine, or with zinc [35]. Liver transplantation, which corrects the underlying hepatic defect in Wilson disease, is reserved for severe or resistant cases.

Penicillamine

Penicillamine was first reported to be effective in treating Wilson disease by Walshe in 1956 and is since the "gold standard“ for therapy. Penicillamine acts by reductive chelation: it reduces copper bound to protein and decreases thereby the affinity of the protein for copper. Reduction of copper thus facilitates the binding of copper to the drug. The copper mobilized by penicillamine is then excreted in the urine. Within a few weeks to months, penicillamine brings the level of copper to a subtoxic threshold, and allows tissue repair to begin. The great majority of symptomatic patients, whether hepatic, neurologic or psychiatric, respond within months of starting treatment. Among neurologic patients, a significant number may experience an initial worsening of symptoms before they get better.

The usual dose of penicillamine is 1 to 1.5 g/day. Initially, this dose will cause a large cupriuresis, but copper excretion later on decreases to 0.5 mg/d. To prevent deficiency induced by penicillamine pyridoxine (vitamin B_6) should be supplemented (50 mg/week). Once the clinical benefit was established, it is possible to reduce the dosage of penicillamine to 0.5 to 1 g/d. A lower maintenance dose will decrease the likelihood of late side effects of the drug.

A major problem of penicillamine is its high level of toxicity. In our series 20% of patients had major side-effects and were switched to other treatments. Other series report even higher frequencies of side effects. There are two broad classes of penicillamine toxicity: direct, dose dependent side effects and immunologically induced lesions. Direct side effects are pyridoxine deficiency, and interference with collagen and elastin formation. The later results in skin lesions like cutis laxa and elastosis perforans serpingiosa. By routine skin biopsies one year after initiation of treatment we found signs of elastic and collagen fiber abnormalities in every patient, but none developed symptomatic skin disease so far. These side effects can be prevented or mitigated by decreasing the dosage of penicillamine. Immunologic mediated side effects include leukopenia and thrombocytopenia, systemic lupus erythematodes, immune complex nephritis, pemphigus, buccal ulcerations, myasthenia gravis, optic neuritis, and Goodpasture syndrome. Immunologic mediated side effects occur within the first three months of treatment and require immediate cessation of penicillamine. To diagnose these side effects as soon as possible, patients should be monitored in weekly intervals during the first six week of therapy. If the drug is well tolerated, control intervals can be gradually prolonged.

Trientine

Trientine is a copper chelator, acting primarily by enhancing urinary copper excretion. Trientine is licensed for treatment of Wilson disease and is now generally available. Experience with trientine is not as extensive as with penicillamine. It seems to be as effective as penicillamine with far fewer side effects. Its efficacy was evaluated in patients with intolerance to penicillamine [36]. Discontinuation of penicillamine resulted in death from hepatic decompensation or fulminant hepatitis in 8 of 11 patients who stopped their own treatment after an average survival of only 2.6 years. In contrast, 12 of 13 patients with intolerance to penicillamine switched to trientine (1 to 1.5 g per day) were alive at 2 to 15 years later. The remaining patient was killed accidentally. However, the efficacy of trientine was not compared with penicillamine as initial treatment of Wilson disease. Uncontrolled anecdotal reports and our own experience indicate, that trientine is a satisfactory first line treatment for Wilson disease. In the early phase of treatment trientine appears to be more potent to mobilize copper than penicillamine, but cupriuresis diminishes more rapidly than with penicillamine. The cupriuretic power of trientine may be disappointing but is sufficient to keep the patient clinically well.

Ammonium Tetrathiomolybdate

This drug has two mechanisms of action. First, it complexes with copper in the intestinal tract and prevents thereby absorption of copper. Second, the absorbed drug forms a complex with copper and albumin in the blood and renders the copper unavailable for cellular uptake. There is very limited experience with this drug. Tetrathiomolybdate appears to be a useful form of initial treatment in patients presenting with neurologic symptoms [37]. In contrast to penicillamine therapy, treatment with tetrathiomolybdate does not result in initial neurologic deterioration. This agent is particularly effective at removing copper from the liver. Because of its effectiveness, continuous use can cause copper deficiency. Besides, bone marrow depression was observed in a few patients treated with this drug.

Zinc

Zinc interferes with the intestinal absorption of copper by two mechanisms. Both metals share the same carrier in enterocytes and pretreatment with zinc blocks this carrier for copper transport (with a half-life of about 11 days). The impact of zinc induced blockade of other copper transport by other carriers into the enterocytes was not studied. Second, zinc induces metellothionein in enterocytes [38], which acts as an intracellular ligand binding metals which are then excreted in the feces with desquamated epithelial cells. Indeed, fecal excretion of copper is increased in patients with Wilson disease on treatment with zinc. Furthermore, zinc also induces metallothionein in the liver protecting hepatocytes against copper toxicity [39,40]. Data on zinc in the treatment of Wilson disease are derived from uncontrolled studies using different zinc preparations (zinc sulfate, zinc acetate) at different doses (75-250

mg/d) [41]. The efficacy of zinc was assessed by four different approaches. First, patients successfully decoppered by d-penicillamine were switched to zinc and the maintenance of their asymptomatic condition was monitored. Most patients maintained a negative copper balance and no symptomatic recurrences occurred. Some patients, however, died of liver failure after treatment was switched to zinc. Stremmel observed the occurrence of severe neurologic symptoms in a 25 year old asymptomatic sibling 4 months after switching from d-penicillamine to zinc [41].

Second groups are symptomatic patients switched to zinc as alternate treatment due to intolerance to D-penicillamine. 16 case histories were published so far. Liver function and neurologic symptoms improved in 3 and 5 patients, respectively. One patient further deteriorated neurologically and improved on retreatment with d-penicillamine. The remaining patients remained in stable condition. Follow-up studies in 141 patients demonstrated that zinc is effective as sole therapy in the long-term maintenance treatment of Wilson disease. In a third group zinc was used as first line therapy. About 1/3 were asymptomatic siblings, 2/3 presented with neurologic or hepatic symptoms. Most patients remained free of symptoms or improved. In 15% neurologic symptoms worsened and improved on d-penicillamine. Three patients died of progressive liver disease. Finally, in a prospective study in 67 newly diagnosed cases the efficacy of d-penicillamine and zinc was similar. This was not a randomized study; every other patient was treated with zinc. Zinc was better tolerated than D-penicillamine. However, two zinc-treated patients died of progressive liver disease.

It is unknown whether a combination of zinc with chelation therapy is useful or not. Theoretically these drugs may have antagonistic effects. Interactions in the maintenance phase of zinc therapy with penicillamine and trientine were investigated by Cu balance studies and absorption of orally administered ^{64}Cu as endpoints. The result on Cu balance was about the same with zinc alone as it is with zinc plus one of the other agents. Thus, there appear to be no advantages to concomitant administration.

Antioxidants

As discussed before, the main mechanism of hepatocellular injury by excess copper is the formation of free radicals resulting in lipid-peroxydation and impaired mitochondrial respiration. Thus, antioxidants, such as a-tocopherol, may be important adjuncts in the treatment of Wilson disease. There are no large experiences with a-tocopherol. A few observations indicate that this therapeutic adjunct may be useful in severe liver disease.

Drug Therapy During Pregnancy

Controversy over prescribing penicillamine in pregnant patients exists due to its possible teratogenic effects. Rare cases of birth defects including hydrocephalus and cerebral palsy have been reported in patients treated with penicillamine for a variety of diseases. However, the overall teratogenic risk of penicillamine is low and there is general support for continuing treatment throughout pregnancy to avoid the risk of relapse in the mother, although the optimal dosage of penicillamine is not known. Trientine appears to be an alternative to penicillamine with no reported teratogenic effects, but the experience with this drug is

limited. The use of zinc in pregnancy has not been associated with any fetal abnormality and possibly has a protective effect from some birth defects. The limited experience with zinc or trientine in pregnancy does not justify a change in drug therapy during pregnancy.

Monitoring Therapy

If a decoppering agent is used for treatment, the compliance can be tested by repeated measurements of the 24 hour urinary copper excretion. This approach is not useful if patients are treated with zinc. If in a compliant patient urinary copper excretion decreases over time and stabilizes at < 500 µg/day, the dose of d-penicillamine can be lowered.

Efficacy of treatment can be monitored by the determination of “free” copper in serum, and depending on the presenting symptoms, Liver disease can be assessed by routine liver function tests. Repeated liver biopsies with measurement of hepatic copper content are not helpful. Improvement of neurologic symptoms can be documented by clinical examination. In addition, some of the MRI abnormalities are fully reversible on treatment. Auditory evoked brainstem potentials are also helpful to document improvement by decoppering treatment.

Liver Transplantation

Liver transplantation is the treatment of choice in patients with fulminant WD and in patients with decompensated cirrhosis. Besides improving survival, liver transplantation also corrects the biochemical defect underlying Wilson disease. However, the role of this procedure in the management of patients with neurological Wilson's disease in the absence of hepatic insufficiency is still uncertain.

Schilsky analyzed 55 transplants performed in 33 patients with decompensated cirrhosis and 21 with wilsonian fulminant hepatitis in the United States and Europe. The median survival after orthotopic liver transplantation was 2.5 years, the longest survival time after transplantation was 20 years. Survival at 1 yr. was 79%. Nonfatal complications occurred in five patients. Fifty-one orthotopic liver transplants (OLT) were performed on 39 patients (16 pediatric, 23 adults) at the University of Pittsburgh. The rate of primary graft survival was 73% and patient survival was 79.4%. Survival was better for those with a chronic advanced liver disease presentation (90%) than it was for those with a fulminant hepatic failure (73%) presentation. In the Mayo clinic series one-year survival ranged from 79% to 87%, with an excellent chance to survive long term. The outcome of neurologic disease following OLT is uncertain. In the retrospective survey four of the seven patients with neurological or psychiatric symptoms due to Wilson's disease improved after OLT. Anecdotal reports documented a dramatic improvement in neurologic function within 3 to 4 months after OLT. In contrast, central pontine and extrapontine myelinolysis and new extrapyramidal symptoms developed in a patient 19 months after OLT. Some patients transplanted for decompensated cirrhosis have had psychiatric or neurologic symptoms, which improved following OLT.

PROGNOSIS

Untreated, symptomatic Wilson disease progresses to death in all patients. The majority of patients will die of complications of advanced liver failure, some of progressive neurologic disease. The overall mortality from Wilson disease treated medically (in most cases by d-penicillamine) has not been assessed prospectively. The mortality in 33 patients followed for 21 years by Scheinberg and Sternlieb was approximately 20. In a German study in 51 patients the cumulative survival was slightly reduced during the early period of follow up but was not different from an age- and sex matched control population after 15 years of observation (96%).

Liver Disease

In general, prognosis depends on the severity of liver disease at diagnosis. In patients without cirrhosis or with compensated cirrhosis liver disease does not progress after initiation of decoppering therapy. Liver function (serum albumin, prothrombine time) improves gradually and will become normal in most patients within 1 to 2 years. In compliant patients treated with d-penicillamine or trientine, liver functions remains stable and no progressive liver disease is observed.

Schilsky followed 20 patients with Wilsonian chronic active hepatitis. Treatment with D-penicillamine was promptly initiated in 19 patients. One refused treatment and died 4 months later. Treated patients received D-penicillamine or trientine for a total of 264 patient-years (median: 14). In 18 symptomatic improvement and virtually normal levels of serum albumin, bilirubin, aspartate aminotransferase, and alanine aminotransferase followed within 1 year. One woman died after 9 months of treatment. Two patients, who became noncompliant after 9 and 17 years of successful pharmacological treatment, required liver transplants.

Table 4. Prognostic index in Wilson disease

	0*	1*	2*	3*	4*
Serum bilirubin (µmol/l)	<100	100-150	151-200	201-300	>301
INR	-1.29	1.3-1.6	1.7-1.9	2.0-2.4	>2.5
AST (IU/L)	-100	101-150	151-300	301-400	>401
WBC (10^9/L)	0-6.7	6.8-8.3	8.4-10.3	10.4-15.3	>15.3
Albumin (g/L)	>45	34-44	25-33	21-24	<21

*= score points, ULN= upper limit of normal.

A score ≥ 11 is associated with high probability of death (without emergency liver transplantation (sensitivity: 93% specificity: 98%, positive predictive value: 88%.).

In patients presenting with fulminant Wilson disease, medical treatment is rarely effective. Without emergency liver transplantation mortality is very high. In a group of 34 patients, Nazer et al developed a prognostic index based on serum bilirubin levels, aspartate aminotransferase activity, and prothrombin time. This score was refined in a large group of

children diagnosed at King´s College in London, UK [42] by including WBC and serum albumin (table 4). A score > 11 was highly predictive of death without transplantation. However, this prognostic score was not validated prospectively. Nevertheless, it is a useful guide to assess short term mortality in the setting of liver transplantation.

Hemolytic Anemia

If diagnosed and treated early, hemolysis subsides within few days after initiation of d-penicillamine therapy. Spontaneous remissions may occur even without treatment but relapse usually within few months. Hemolysis associated with active liver disease may progress to fulminant Wilson disease rapidly.

Neurologic Disease

Patients presenting with neurologic symptoms have a better prognosis than those presenting with liver disease. The prognosis for survival is favourable [43], provided that therapy is introduced early.

In Brewer´s series, 2 out of 54 patients died due to complications which were attributed to their impaired neurologic function [44].

Neurologic symptoms are partly reversible. Improvement of neurologic symptoms occurs gradually over several months. Initially, neurologic symptoms may worsen, especially on treatment with d-penicillamine. In some patients neurologic symptoms disappear completely, and abnormalities documented by evoked responses or MR-imaging may completely resolve within 18 to 24 months. Brain function was assessed by repeated recording of short latency sensory potentials, auditory brain stem potentials and cognitive P300 evoked potentials in 10 patients followed prospectively after diagnosis for 5 years. [45]. Electrophysiological and clinical improvement was observed as early as 3 months after initiation of chelation therapy and continued until final assessment after 5 years. Three patients became completely normal but residual symptoms were detectable in 7. Czlonkowska et al [46] studied 164 patients diagnosed over an 11 year period. Twenty died during the observation period. The relative survival rate of all patients in our group was statistically lower than in the Polish population. The main cause of death was diagnosing the disease at an advanced stage, but in six patients presenting with mild signs disease progressed despite treatment. There was no difference in mortality rate in patients treated with d-penicillamine or zinc sulphate as initial therapy.

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Chapter VIII

GAUCHER DISEASE

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ABSTRACT

Gaucher disease (GD), the most common lysosomal storage disease, is caused by mutations in the β-glucocerebrosidase gene, and results in accumulation of glucosylceramide in macrophages ("Gaucher" cells) of the spleen, liver, and bone marrow. Since the advent of enzyme replacement therapy (ERT) for Gaucher disease a decade and a half ago, the quality of life of patients has improved substantially: symptomatic patients have benefited from reduction in hepatosplenomegaly and improvement in anemia and thrombocytopenia. While there are broad correlations between specific mutations, i.e. the genotype, and the clinical course, i.e. the phenotype, (such as between the most common "Jewish" mutation N370S with type I, non-neuronopathic GD or homozygosity of the L444P mutation and type II or type III, neuronopathic, GD), predictions based on genotype are imperfect, and hence researchers are still trying to identify modifiers and effectors that impact clinical heterogeneity. In assessing a patient for GD, in addition to the enzymatic assay to diagnose GD and the surrogate markers chitotriosidase and CCL-18, plus evidence of anemia and thrombocytopenia, other laboratory tests may not be within normal ranges; liver function tests are usually abnormal only in severely affected patients. Visceral imaging (liver and spleen) is based on ultrasonography, CT or MRI.

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INTRODUCTION

Definition and Epidemiology

Gaucher disease (GD) was described in 1882 by a French medical student, Philippe Gaucher, who assumed that the large cells which today bear his name were a manifestation of a primary splenic neoplasm. Today it is apparent that GD, the most prevalent sphingolipid storage disorder, is caused by deficiency of the lysosomal enzyme β-glucocerebrosidase, leading to the accumulation of glucocerebroside, in macrophages [1]. These "Gaucher cells", which are filled with undegraded substrate, accumulate in and impact the function of many organs and tissues, but initially and universally, the liver, the spleen, and the bone marrow.

Although there are some enclaves with high mutation incidence such as in the Norbottnian province in Sweden, GD has an ethnic predilection primarily among Ashkenazi Jews, where the carrier frequency is 1:17 (i.e. a prevalence of about 1:850 live births) [1]. In the general population, the estimated frequency is in the range of 1:50,000 to 1: 1,000,000 [2].

Classification into three Clinical Forms

GD is characterized its considerable phenotypic heterogeneity with a complete spectrum of clinical morbidity. In its mildest form, there are totally asymptomatic individuals (no signs, no symptoms, and with normal values of almost all specific laboratory parameters) whose diagnosis is made incidentally, for example, during routine genetic screening [3]. At the other extreme, with the most severe presentation, is a neonatal variant with severe multi-organ involvement with brain damage, hydrops fetalis and ichthyosis, with death occurring either *in utero* or within the first 2 days of life [4].

Today one can appreciate that GD is in fact a continuum of clinical entities; however, traditionally, the disease was divided into three forms based on the absence (type I) or presence and severity of central nervous system involvement (types II and III). This phenotypic classification actually preceded identification of the defective enzyme as the underlying etiology of GD.

Type I, also known as “adult” or “chronic" form is by definition non-neuronopathic; it is the most prevalent form, accounting probably for more than 95% of the world´s patients, with expression at any age from childhood to old age. There is a high prevalence among Ashkenazi Jews.

Types II, the “infantile” or “acute" neuronopathic form and type III, the “juvenile” or “sub-acute" neuronopathic form, are panethnic, but relatively rare among European and American Caucasians (estimated frequencies according to the International Gaucher Registry are >1% and >5% for types II and III, respectively; [5]), but putatively more common than type I in Asia or in Arab countries (e.g., Egypt), which are under-represented in the Registry and where epidemiological data are unavailable. Further subclassifications are available [6], but clinically the most used is the one named above.

Molecular Biology and Pathophysiology

The variability in clinical features is related in part to the many (>250) mutations identified to date in the glucocerebrosidase gene [7]. While there are broad correlations between specific mutations, i.e. the genotype, and the clinical course, i.e. the phenotype, (such as between the most common "Jewish" mutation N370S with type I or homozygosity of the L444Pmutation and neuronopathic disease; [8]), predictions based on genotype are imperfect. Several reports have addressed the intra-familiar heterogeneity of siblings with the same genotype, underscoring the importance of environmental and other genetic factors ("modifier genes"). Inflammatory cytokines may be candidate "modifiers" and have been studied relative to GD severity, but there have been only few studies with statistically significant results [9] supporting the relation between a specific polymorphic change in a gene encoding a cytokine, such as IL-6, which is known to be elevated in GD [10], and clinical disease manifestation.

A recent study by Ron and Horowitz suggests dysfunction of the endoplasmic reticulum (ER) as a new cellular pathological process in GD [11], playing a critical role in the clinical course of the disease. These investigators have shown that mutations which lead to glucocerebrosidase misfolding induce trafficking of the mutated protein that is either disturbed or acceptable, and because of this, phenotype may be more severe or mild, respectively [11]. This hypothetical construct, that looks not only at accumulation of the undigested glycolipid, but also at the proteotoxic effect of the misfolded mutant enzyme in the ER, has led to the development of a new class of pharmacological chaperones [12].

In liver, infection and inflammation lead to an acute phase response and lipoproteins become enriched in ceramide, glucosylceramide, and sphingomyelin, enhancing uptake by macrophages [13,14]. GD, on the other hand, is characterized by primary accumulation of glucosylceramide that triggers a chronic inflammatory state, that is, admittedly, still poorly understood [15-17]. Importantly, however, in animals given [^{14}C]-labelled glucosylceramide intravenously, glucosylceramide is predominantly stored in the liver [18]. The half-life of glucosylceramide is about 3.5 days, with predominant excretion via bile. In livers from rats who were treated with conduritol-B-epoxide, an inhibitor of glucocerebrosidase, or who were injected with glucosylceramide emulsion, protein, lipid and DNA content is increased. It is not clear whether this protein retention is due to increased protein synthesis or to decreased protein degradation [19].

Although it is well known that GD can lead to cirrhosis [20], hepatocellular carcinoma without pre-existing cirrhosis [21] and cholelithiasis [22], the role of glucosylceramide for bile formation and bile composition is not fully understood.

Diagnosis

The gold standard for the diagnosis of GD is demonstration of decreased β-glucocerebrosidase activity in peripheral blood samples. In case of leucocytopenia, this assay can also be performed in fibroblasts. Despite widespread availability of this simple biochemical assay since 1970, it is only rarely used when GD is first considered in the

differential diagnosis of a patient; unfortunately, too, physicians still refer patients for a bone marrow examination in order to the identify Gaucher cells in the aspirate [23]. Indeed, the symptoms of hepatosplenomegaly with signs of pancytopenia, often induce physicians to recommend bone marrow aspiration, liver and even spleen biopsy which may lead to an unwarranted splenectomy. It is important to emphasize that invasive diagnosis is not necessary. Today enzymatic diagnosis is performed in conjunction with PCR-based DNA mutation analysis. As the availability of sequencing services increases, it is to be expected that in the future each patient will have a complete sequence of the relevant gene, allowing not only the identification of the rare private mutations, but also avoidance of errors related to more complex mutations that are currently missed due to pitfalls of the current methodology [7].

In the past decade, surrogate markers, chitotriosidase and CCL-18, have been added to the diagnostic work-up of GD; both markers are highly elevated in patients with GD, to a level that they provide a "quality control" for the glucocerebrosidase assay [16,24]; in addition they are useful for monitoring the clinical course of disease progression, relapse, or response to specific therapy.

CLINICAL PRESENTATION WITH EMPHASIS ON THE LIVER

Signs and Symptoms

While phenotypic heterogeneity is a hallmark of GD, invariably most patients of all ages present with symptoms, signs or laboratory findings related to splenomegaly and its related hypersplenism. In general, thrombocytopenia is more pronounced than anemia [6] (leucopenia is rarely severe and by itself has not been associated with increased tendency to bacterial infections), and repeated episodes of epistaxis or excessive bleeding after dental procedure, delivery or surgery, are among the common presenting features. Splenomegaly as an incidental finding during an intercurrent illness or routine physical examination is rather common, as is pancytopenia detected upon routine blood count. Splenomegaly is more pronounced than hepatomegaly, and in addition to the associated anemia and thrombocytopenia, may be associated with linear growth retardation in children, with early satiety and/or abdominal discomfort. In more severe cases, complications such as splenic infarction or subcapsular bleeding following trauma or extraordinary physical effort may occur.

An experience-based axiom is that the earlier the age of presentation, the more severe the clinical course. Ashkenazi Jewish patients, due to the high prevalence of the "mild" mutation N370S, tend to have a milder phenotype relative to non-Jewish patients, and there may be specific ethnic groups, such as the Japanese [25], who tend to develop a particularly severe course (including in the case of L444P homozygotes where some Japanese had been thought to have type I disease but, with better analyses, like other ethnicities have neuronopathic disease [26]. Accordingly, the mean age of diagnosis at the International Gaucher Registry, the largest database of patients with GD (albeit, with ascertainment bias towards more severely affected patients since the emphasis is on patients receiving enzyme replacement

therapy), in patients with the N370S/N370S genotype (mostly Ashkenazi Jews) was 27.2 years [5], whereas in Japan >60% of type I patients experienced onset of GD signs/symptoms at <5 years [25]. Bone pains are less frequent at presentation, although there are patients whose first manifestation of GD is acute bone crisis. These episodes are often predictive of a more severe clinical course because of skeletal complications. Prior to the advent of enzyme therapy, bone crises typically developed within a short period after splenectomy. Similarly, many skeletal complications are seen in splenectomized patients [27]. In more severely affected patients, bone pain is present at more than half of the patients [28].

In communities where genetic screening programs includes GD, the vast majority of patients are diagnosed when they are asymptomatic. Moreover, because of early diagnosis of GD, patients may present with atypical manifestations; however, skeletal involvement remains an important symptom of clinical expression even in the era of enzyme replacement. Destruction of the bone is a major feature of GD and results from expansion and activation of Gaucher cells within the bone marrow. Major bone complications comprise bone crises, pathological fractures (mainly of the ribs), osteolytic lesions and avascular necrosis of large joints (Figure 1), usually the hips, knees or shoulders.

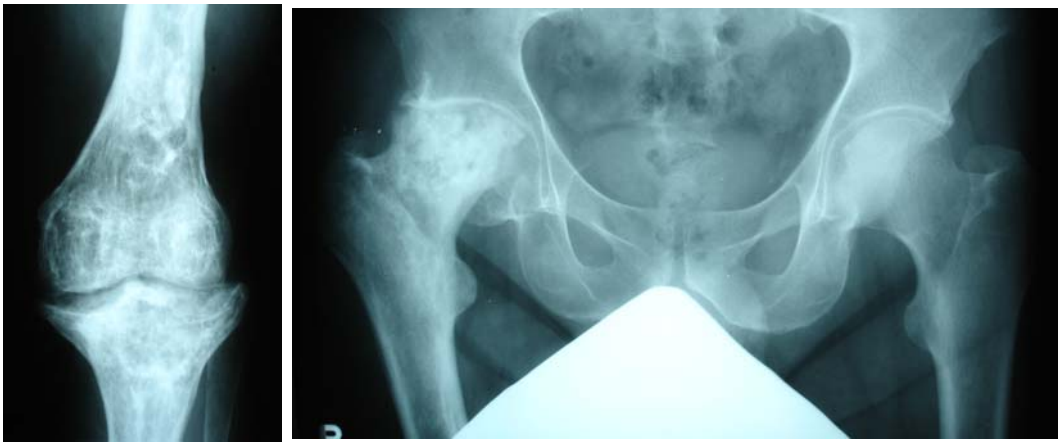

Figure 1. Typical skeletal lesions. Erlenmeyer flask deformity of the distal femur with lytic lesions (A) and avascular necrosis of the right hip joint (B).

Pulmonary features include interstitial lung disease and pulmonary hypertension, both rare and usually with poor prognosis. The availability of ERT has changed the natural history of GD, and when administered prior to the development of irreversible bone lesions, secondary skeletal and pulmonary complications will hopefully be prevented.

Liver manifestations other than hepatomegaly *per se* are infrequent, and tend to develop in patients with other signs of severe disease, including after splenectomy. In splenectomized patients, the liver may be massively enlarged, and may be palpable in the left lower quadrant. Liver function tests may be normal even in patients with marked hepatomegaly, and in the more severely affected patients, abnormal liver enzyme tests, along with hyperbilirubinemia, low albumin and abnormal prothrombin time (PT) may be detected. Cirrhosis with portal

hypertension may develop in such severely affected patients, and this in turn may lead to liver failure and the need for liver transplantation [29]. Hepato-pulmonary syndrome, although a rare entity, has also been reported with the classic triad of advanced liver disease, arterial deoxygenation and intrapulmonary vascular dilatation [30].

Laboratory Findings

In working up a patient for GD, in addition to the enzymatic assay to diagnose GD and the surrogate markers chitotriosidase and CCL-18, plus evidence of anemia and thrombocytopenia, other laboratory tests may not be within normal ranges. These laboratory tests may be grouped as:

- complete blood count and coagulation profile,
- inflammatory markers,
- biochemical and serological abnormalities and
- other surrogate markers.

The majority of these abnormalities may be detected in routine studies whereas others may require the expertise of dedicated research laboratories.

Complete Blood Count and Coagulation Profile

Anemia and thrombocytopenia are very common in GD; both may be caused by hypersplenism *per se* (increased sequestration of blood cells within the enlarged spleen), but both may have other causes. Anemia may be due to iron deficiency (caused either by excessive bleeding or by altered iron metabolism), vitamin B_{12} deficiency, autoimmune haemolysis or bone marrow failure. In most of the patients the mean corpuscular volume (MCV) tends to be on the high side, primarily because of liver involvement and an increased fraction of young erythrocytes. Immune thrombocytopenia and marrow failure may also account for low platelet counts, while thrombocytopathy (abnormal platelet aggregation and/or adhesion, as functional defects) may be an additional cause for bleeding tendency [31]. The latter may be due to diminished clotting factors and deficiencies of factors II, V, VII, VIII, X, XI and XII have been described in as many as 40% of adult patients with GD [32]. Increased clearance through the enlarged spleen, increased activation of coagulation as well as fibrinolysis, (leading also to elevated D-dimers) have all been implicated as causes for these deficiencies, while factor XI deficiency may be due to another genetic defect commonly found among Ashkenazi Jews [33].

Inflammatory Markers

Significant elevations in fibrinogen, erythrocyte sedimentation rate and C-reactive protein, indicative of a low-grade inflammatory profile, have recently been reported. Nonetheless, these inflammatory markers do not necessarily correlate with disease severity, and they do not improve with ERT [34], unlike the polyclonal hyperglobulinemia, another feature of inflammation, which does decrease with ERT [35]. Pro-inflammatory cytokines,

such as serum IL-6 and IL-10 are elevated in GD, are probably secreted by the Gaucher cells, and their levels also decrease with treatment [34].

Biochemical and Serological Findings

Routine biochemical panel may be either within the normal range, or may show mildly abnormal liver function tests (only in the few patients with hepatic fibrosis or cirrhosis are these tests severely abnormal: see below). Blood urea nitrogen (BUN) and serum creatinine levels tend to be lower than the normal range (but generally without clinical significance) as are total cholesterol, LDL- and HDL-cholesterol [36]. It has been shown that these decreased levels are due to the reduced apoprotein levels that are part of the structure of the HDL and LDL particles, apo-B and apo-A_1, respectively [36], while apoE is increased. Interestingly, mildly elevated aminotransferases may eventually respond to therapy [37]. Another study has shown that patients with Gaucher disease have decreased plasma taurine levels and that ERT might correct this [38]. Taurine is an osmolyte capable of exerting chaperone-like functions in the liver although it is as yet unclear whether decreased taurine availability in liver could be a cofactor in permanent activation of the glucosylceramide-storing macrophages in GD.

Parameters of bone metabolism, either bone formation or bone resorption, have shown conflicting results, and are therefore not used clinically. Some patients may have abnormalities in serological parameters, e.g. NT-brain natriuretic factor, which is correlated with pulmonary hypertension even in GD [39].

Additional Surrogate Markers

Plasma chitotriosidase and CCL18 levels are surrogate markers, show elevated levels in patients versus control subjects, correlate with disease severity, and are reduced concomitant with ERT [16]. The simplicity of the new assays and its reliability will probably obviate the use of the traditional markers, such as angiotensin converting enzyme (ACE), tartrate-resistant acid phosphatase (TRAP), hexosaminidase, ferritin, which, although elevated in GD, are nonetheless not as sensitive and are less good correlates of clinical severity [40]. Other markers of macrophage activation, such as sCD163, cathepsin K, and neopterin [40,41], have all been described in research labs but are not used clinically.

Diagnostic Imaging

The radiological findings in GD may be classified into skeletal, pulmonary, and visceral, according to the organs involved. Most patients, including the mildly affected ones, will usually show the “Erlenmeyer flask deformity” of the distal femora on plain x-rays, which may also have some evidence of sclerotic and/or lytic lesions; patients with more severe disease may demonstrate osteoporosis, avascular necrosis, or pathological fractures [42]. A more sensitive imaging of the bones is achieved with magnetic resonance imaging (MRI) where extensive changes of the bone marrow can be seen that were not evident on plain X-ray [43].

Chest X-ray is usually normal, but patients with severe disease may evince a range of pulmonary abnormalities that may be interstitial (with ground-glass appearance on

computerized tomography; CT) or vascular, i.e. pulmonary hypertension [44]. Often the choice of imaging is critical to identification of pathology [44].

Visceral imaging (liver and spleen) is based on ultrasonographic study, CT or MRI. The main abnormality other than organomegaly, is the presence of space-occupying-like lesions, that may be identified as hypo- or hyperechoic or mixed lesions [45]. These lesions are found more frequently in the spleen [46,47] than in the liver, and more often in adults than in children [48]. The importance of their identification is mainly to differentiate them from tumours [49,50], in particular hematological malignancies, which have been reported to be more common in patients with GD relative to the general population (see below). Often pseudotumours inside or adjacent to the liver have been observed. They are usually mistaken for solid lesions and result in a thorough diagnostic work-up to exclude a hepatic malignancy or a metastasic process. Usually, biopsy reveals a "Gaucheroma", which is a tumour-like accumulation of Gaucher cells, that can occur in almost any region of the body [51]. Imaging of these intra- or para-hepatic lesions may mimic hepatocellular carcinoma, metastases or even focal nodular hyperplasia, requiring an experienced eye for diagnosis (Figure 2).

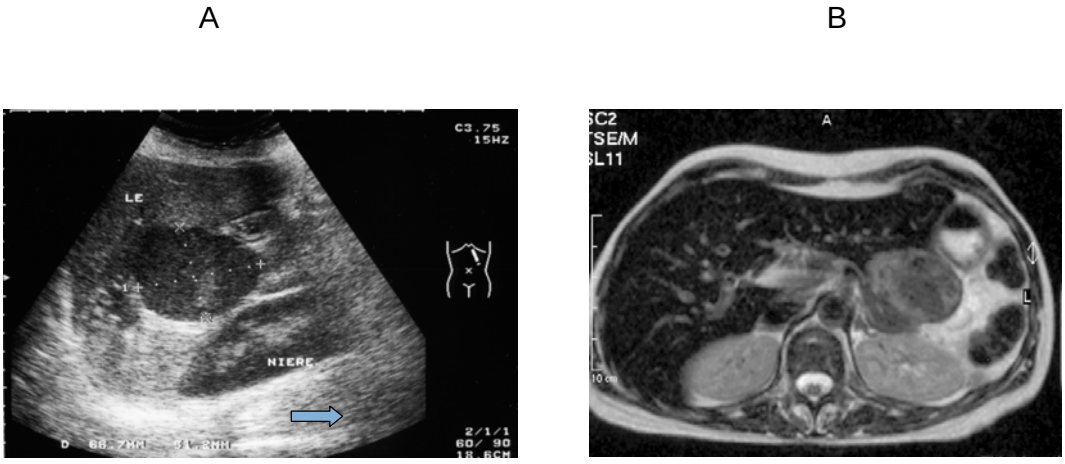

Figure 2. Gaucher cell pseudotumour adjacent to the liver. Typical ultrasound (A) and MR (B) aspect of a pseudotumour-like parahepatic, hyperechoic, hypointense lesion, suspicious of a solid lesion like HCC or metastasis. Biopsy revealed no hepatocytes, but only Gaucher cells, see also [22].

In addition to the role of radiological imaging in the evaluation of the patients with GD at baseline, radiological imaging is frequently used for follow-up and to document the response to treatment, disease-specific and otherwise. While both CT and MRI provide accurate organ volume estimations, they are expensive and relatively rare resources. In addition, CT involves considerable amounts of radiation, if used repeatedly (which is a particular concern in children and young adults) and MRI may require general anaesthesia in young children and claustrophobic patients of all ages because it is unpleasant and requires immobilization for a longer time period. Therefore, for routine follow-up we prefer to use ultrasound, with CT in abeyance for specific questions, while MRI volumetric assessment is the preferred modality in the context of clinical trials in adults. MRI and bone densitometry are useful for

monitoring bone response or disease progression in treated and untreated patients, respectively.

Liver Diseases Associated with Gaucher Disease

Viral Hepatitis and Autoimmune Hepatitis

Viral hepatitis may develop in patients with GD, but there is very little information about these co-morbidities in the literature. In the referral clinic at Shaare-Zedek Medical Centre in Jerusalem, 28 patients out of >550 patients had positive serological markers for active hepatitis B or C (11 and 12 patients respectively) or both (5 patients), the majority of whom were probably infected following blood transfusions given during surgical procedures, prior to the availability of reliable tests to detect viral hepatitis in blood donors. Yet, only a handful of patients developed clinically significant hepatitis with the need to receive specific HCV therapy [52].

Liver fibrosis and cirrhosis are rare in GD. In an attempt to investigate the mutual impact of viral hepatitis and GD on each other, Margalit and Ilan have recently discovered that patients with GD have an altered humoral and cellular immune profile, including a markedly increased number of peripheral blood killer cells (NKT) cells [53]. In order to investigate potential benefit for patients with GD, which they hypothesized was related to elevated intracellular levels of glucocerebroside, they showed that administration of β-glucosylceramide resulted in marked amelioration of concanavalin A-induced hepatitis in mice, a model in which NKT cells are key mediators of hepatic damage [54]. These preliminary observations may explain, in part, the relative rarity of HCV-related cirrhosis among patients with GD, and also provide another example where studies of patients with a rather rare inherited disorder, may have ramification to larger numbers of patients suffering from other (in this case, immune-mediated) disorders. In fact, Phase I clinical studies with β-glucosylceramide are pending for patients with non-alcoholic steatohepatitis (NASH) and Crohn's disease.

The hyperactivity of the immune system in GD is also manifested by high prevalence of polyclonal hypergammaglobulinemia and an increased incidence of monoclonal gammopathies. High titers of natural, polyspecific, non-pathogenic autoantibodies in the sera of GD patients have been demonstrated but these were not correlated with the immunoglobulin levels [55]. In addition, there is an impression of increased prevalence of autoimmune disorders in GD, but no formal study has substantiated this anecdotal experience. In the experience of the authors, several autoimmune disorders, such as autoimmune haemolytic anemia [56], immune thrombocytopenia and Hashimoto thyroiditis, may be more common in patients with GD.

With regard to the liver, a single patient with GD and autoimmune hepatitis is known to us, who required courses of steroids. Subsequently, ERT was given in the hope of preventing osteoporosis in an osteopenic patient. There is also some hope that, if there is a relation between the metabolic defect of GD and the development of an autoimmune disorder, that the reduction in storage cells and their secretory products may have a beneficial effect. There is also a case report of chronic active hepatitis in a patient with GD prior to the ERT era [52];

the clinical course of remissions and exacerbations of the disease activity was typical of "autoimmune" chronic active hepatitis and seemed unaffected by the coexistence of GD. Steroid and immunosuppressive treatment resulted in prompt resolution of the chronic hepatitis, with no apparent inimical impact on bones.

Liver Cirrhosis and Portal Hypertension

In 1981, in a study of the clinical and liver histopathological observations among 25 patients with GD, three cases with cirrhosis were noted [57]. Two single cases were reported in 1964, with a few others having been reported later, mainly in case reports of orthotopic liver transplantation. It is of interest that in our single case of documented histological evidence of liver cirrhosis in GD (which was diagnosed together with hepatocellular carcinoma), the patient was positive for hepatitis C virus. Almost all the reported patients had been splenectomized at an early age, and all suffered from severe, progressive, multi-organ involvement. In a pathological study of 275 patients from 1982 by Lee [58], end stage liver failure and/or bleeding esophageal varices were among the causes of death from type I GD; whereas in a small series of 5 fatal cases of type I GD from Japan (all splenectomized) four patients suffered from liver cirrhosis [59]. It is to be hoped that in the era of ERT when splenectomy is not part of the management of patients with GD, and when liver function can be kept within the normal range, there will be no new cases of cirrhosis related to GD.

Hepatocellular Carcinoma

Increased incidence of malignancies among patients with GD has been suggested in the literature of the past two decades: initially because of individual case reports, but subsequently in a study from 1993, which showed that 10 of 48 (20.8%) patients with GD had developed a malignancy, as compared with 35 of 511 (6.8%) among the control group [60]. Because this latter study and other small series noted in the literature suffered from some methodological flaws and because the concern of a predilection for cancer was a real concern for patients with GD, two independent groups, a single referral clinic in Jerusalem with more than 500 patients and the International Gaucher Registry, have studied this assertion. The conclusions of both studies were that with the possible exclusion of multiple myeloma, there is no increased incidence of any malignancy among patients with GD [61,62]. Nonetheless, an even more recent collaborative study from The Netherlands and Germany identified 14 non-Ashkenazi patients with GD (out of 131 patients) who developed a cancer, implicating an increased risk of 2.5 for all cancers and an increased risk of 12.7 for hematologic malignancies relative to control population [62]. It is noteworthy that the two most common malignancies in the above Dutch-German collaboration were multiple myeloma and hepatocellular carcinoma in the absence of preexisting cirrhosis [63]. It may be speculated that the obvious conflicting results are due to differences in age (younger median age and potential underreporting in the International registry database [62]) or milder disease severity (more Ashkenazi Jewish patients in the Jerusalem study [61]). A single case of hepatocellular carcinoma was also seen in the Jerusalem clinic (unpublished), but the liver had a cirrhotic appearance (Figure 3). Comparable to the three patients noted in the literature, the Jerusalem patient was also a splenectomized patient with very severe Gaucher disease (including long-standing pulmonary involvement), who had been treated with ERT for more

than 11 years. While we believe that ERT prolongs the lives of severely afflicted patients (i.e. hepatocellular carcinoma can develop in any patient with cirrhosis of liver), others have speculated that ERT itself might be the causative factor [64]. There is a case report prior to the era off ERT of a patient with GD and HBsAg-positive cirrhosis who was found at autopsy to have hepatocellular carcinoma suggested by antecedent ultrasound [65], and there are reports after the era of ERT [66]. Given the grave prognosis of a late diagnosis of hepatocellular carcinoma, α–fetoprotein and a comprehensive hepatic ultrasound should be part of the routine annual follow-up for at risk patients. Finally, an aggressive diagnostic approach (liver biopsy) should be taken in patients with emergent evidence of hepatic lesions on ultrasound [67].

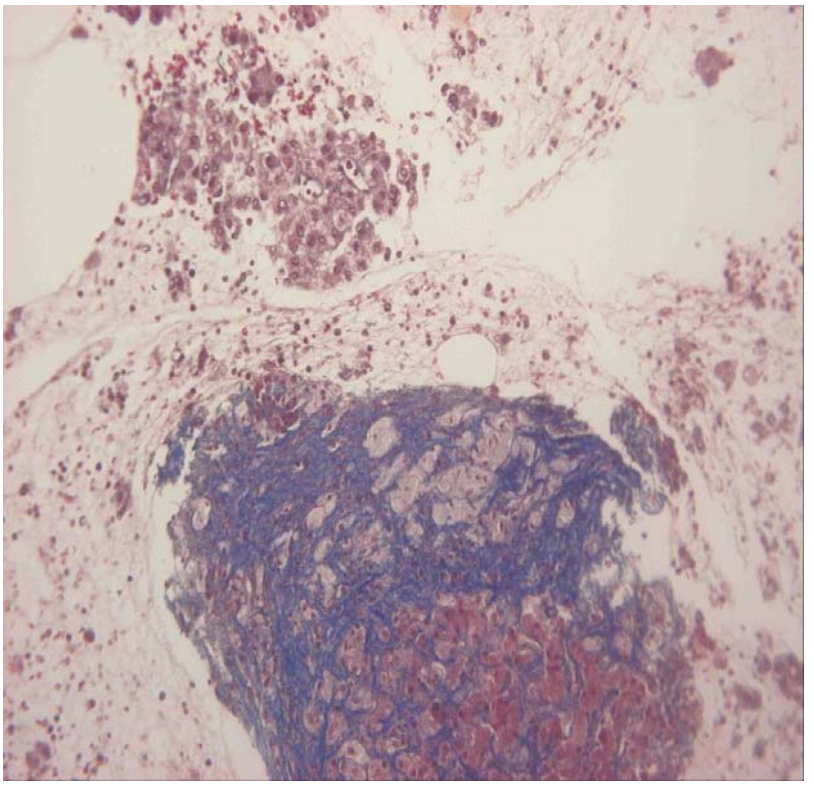

Figure 3. Gaucher cells showing cirrhosis and a hepatocellular carcinoma. A liver biopsy from a Gaucher patient showing massive cirrhosis and associated hepatocellular carcinoma.

Other Hepato-Biliary Complications

Other possible liver complications include cases of severe liver fibrosis without evidence of cirrhosis, non alcoholic steato-hepatitis (NASH), amyloidosis and neonatal hepatitis. Two studies suggested increased prevalence of gallstones in patients with GD (including among male patients); various factors may contribute to gallstone formation in these patients, including anemia, prior splenectomy, hepatic involvement and increased biliary excretion of glucosylceramide [68,69].

EFFECT OF THERAPY: ERT AND SRT

Therapeutic goals in Gaucher disease are:

- Normalisation of linear growth and unimpaired cognitive development in children
- Restoration quality of life and functional mobility
- Prevention of progression of skeletal involvement
- Prevention of bone complications
- Improvement of atypical manifestations (ocular, cardiac, pulmonary)
- Freedom or relief from pain
- Discontinuation of analgesics
- Normalization of organ volumes
- Prevention of bleeding
- Normalization of leucocytes, hemoglobin and platelet counts

Improvement in the hematological parameters and reduction in organomegaly are usually achieved within approximately the first two or three years of therapy. Both placenta-derived and recombinant ERT [70,71] and substrate reduction therapy (SRT) [72] have been shown to be efficacious in meeting these goals, although SRT is less potent and has several side effects. Thus, with the passage of a decade and a half of therapeutic options for haematological and visceral normalization, the goals of therapeutic intervention for GD now devolve on its ability to impact bone pathology and other more severe manifestations of GD. To date it appears as if some complications of bone and lung and brain are virtually irreversible, and therefore the emphasis is on preventing these complications from happening by early specific treatment.

Reduction of liver volume has been an important outcome measure in the clinical trials that led to approval of ERT [70,71] and SRT [72] for type I GD. The choice of the liver volume is logical: it is always enlarged in symptomatic patients; unlike the spleen which had been removed in many patients, the liver is present and accessible; unlike haemoglobin and even platelets, it is rarely affected by confounding factors and concurrent and intercurrent diseases; and unlike the skeletal features which are so slow to respond, the liver is expected to show significant reduction within 6 months [73]. In the minority of patients with abnormal liver function tests, specific therapy will improve these results [74]. Some patients may not respond with significant reduction in liver volume within the first year, but these patients usually evince a very dramatic reduction in splenic volume, so that the reduction in hepatomegaly seems to occur later. It is virtually universal that specific therapy will induce reduction of hepatomegaly to approximately normal size.

If there is no response of the liver at all (no reduction in hepatomegaly, no improvement in liver function tests) one should look for an additional pathological process, especially in splenectomized patients. Examples from the literature and from the authors' unpublished experience include severe liver fibrosis, an associated autoimmune hepatitis, or hepatocellular carcinoma. Patients with severe GD at baseline, who have already developed liver cirrhosis, are also less likely to achieve significant (hepatic) benefit.

Data from the International Gaucher registry show a 20-30% reduction in liver volume in one to two years and up to 40% in five years with ERT [75]. The improvement in spleen size with ERT is even more dramatic, with decreases of 30% and 50% after one and two years, respectively [75]. Based on the International Gaucher registry's cumulative experience in more than 1000 patients, therapeutic goals have defined these values as indicators for satisfactory response [73]. After the first 2-5 years on ERT, most patients will achieve their optimal response, they will plateau and further change in dose will have little or no clinically important effect [75]. At this time point, dose reduction with an eye towards a maintenance regimen should be considered.

The clinical trials of SRT with miglustat (Zavesca®, Actelion Pharmaceuticals, Basel, Switzerland) also used reduction in hepatomegaly as outcome measure of the clinical trials. Although the results were not as dramatic as those reported with ERT, they were statistically significant as early as 6 months [72,76]. In addition, the reduction in hepatomegaly continues beyond the first 2 years of therapy, plateauing in a manner comparable to that seen with ERT but over a more protracted course. However, one should bear in mind, that the clinical trials of SRT enrolled patients with mild to moderate disease severity, and hence the degree of hepatomegaly was not as significant as that reported in the patients who were enrolled in the clinical trials with the ERT (the greater the initial size, the greater the initial reduction with specific therapy [71]). Future studies may allow better comparisons between ERT and SRT.

Therapeutic options in the future include the options of gene therapy, stem cell therapy, and chaperone-like substances, but today, none has succeeded as a viable alternative to currently available commercial therapeutic modalities.

CONCLUSIONS

GD is a multi-system disease whose visceral manifestations can be treated with enzyme replacement. Hepatosplenomegaly is the most common pathology seen by imaging. The main abnormality other than organomegaly, is the presence of space-occupying-like lesions, that may be identified as hypo- or hyper-echoic or as mixed lesions. These lesions are found more frequently in the spleen than in the liver, and more often in adults than in children. Liver fibrosis and cirrhosis are rare in GD. In the single case of documented histological evidence of liver cirrhosis in GD in our combined experience (which was diagnosed together with hepatocellular carcinoma), the patient was positive for hepatitis C virus. Almost all the reported patients with cirrhosis had been splenectomized at an early age, and all suffered from severe, progressive, multi-organ involvement. In a pathological study of 275 patients, end stage liver failure and/or bleeding esophageal varices were among the causes of death from type I GD; whereas in a small series of 5 fatal type I GD from Japan (all splenectomized), four patients had liver cirrhosis. Other possible liver complications include cases of severe liver fibrosis without evidence of cirrhosis, non alcoholic steatohepatitis (NASH), amyloidosis and neonatal hepatitis. Two studies suggested increased prevalence of gallstones in patients with GD. Data from the International Gaucher registry show a 20-30% reduction in liver volume in one to two years and up to 40% in five years with ERT; similarly, clinical trials of substrate reduction therapy with miglustat used reduction in

hepatomegaly as an outcome measure and although results were not as dramatic as those reported with ERT, they were statistically significant as early as 6 months.

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Chapter IX

THE CLINICAL FEATURES AND PATHOBIOLOGY OF ALPHA$_1$-ANTITRYPSIN DEFICIENCY

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ABSTRACT

α_1-Antitrypsin deficiency most commonly results from the severe Z deficiency allele (Glu342Lys). The point mutation causes an aberrant conformational transition within the α_1-antitrypsin molecule and the formation of polymers that are retained within the endoplasmic reticulum of hepatocytes. It is these polymers that underlie the PAS positive inclusions that are the characteristic feature of the disease. The clinical spectrum of liver disease in α_1-antitrypsin deficiency is broad, ranging from mild abnormalities in liver function tests to cirrhosis and hepatocellular carcinoma. Both male gender and obesity are linked to poor prognosis. Other conditions associated with α_1-antitrypsin deficiency include emphysema, panniculitis and vasculitis. Smokers are particularly susceptible to the development of emphysema due to the unopposed action of proteases on the pulmonary parenchyma causing tissue destruction. Treatment options for the hepatic complications of α_1-antitrypsin deficiency include symptomatic support, reduction of portal hypertension and ultimately liver transplantation. The disease does not recur in the transplanted organ. Future treatment strategies may include inhibiting α_1-antitrypsin polymerization and gene therapy.

INTRODUCTION

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α_1-Antitrypsin is the most abundant protease inhibitor in the circulation and the archetypal member of the serine protease inhibitor (serpin) superfamily [1]. It is a 52kDa glycoprotein secreted by hepatocytes and to a lesser extent, by bronchial epithelial cells, macrophages and the intestinal epithelium [2]. By forming an irreversible complex with locally released neutrophil elastase, α_1-antitrypsin protects the connective tissues from proteolytic attack. This is most important within the lung as genetic deficiency of α_1-antitrypsin is associated with the development of early onset panlobular emphysema [2,3]. In fact, deficiency of this protein has been associated with many different pulmonary syndromes including chronic bronchitis [4,5], asthma [4,6], bronchiectasis [4,7-9] and pulmonary vasculitis [4,10-12], although the evidence for a link with bronchiectasis is poor.

The role of α_1-antitrypsin in the pathogenesis of chronic liver disease is quite different as this is caused by protein overload rather than plasma deficiency. In this chapter we review the epidemiology and clinical features of α_1-antitrypsin deficiency associated liver disease and demonstrate how understanding the molecular mechanism will allow the development of novel therapeutic strategies.

THE ALLELIC VARIANTS OF ALPHA$_1$-ANTITRYPSIN

Many allelic variants of α_1-antitrypsin have been described [2,13]. They are inherited in a co-dominant fashion and classified according to their migratory profile on isoelectric focusing analysis. The normal allele is denoted as M and the commonest deficiency variants, S and Z, result from point mutations in the α_1-antitrypsin gene, which is located at 14q32.1 within the SERPIN supergene cluster [14]. The S variant (Glu264Val) results in a 40% deficit in plasma protein levels [15] but is not linked to any clinical disorder. The Z variant (Glu342Lys), in contrast, results in severe plasma deficiency and progressive clinical disease. Other mutations causing severe plasma deficiency include the Siiyama (Ser53Phe) and Mmalton (deletion of 52Phe) variants. A milder form of plasma deficiency is caused by the I allele (Arg39Cys). The α_1-antitrypsin phenotypes known to be associated with clinical liver disease are shown in Table 1.

Table 1. α_1-antitrypsin phenotypes associated with liver disease [16].

Phenotype	Risk of liver disease
ZZ	+++
SZ	++
MZ	+

There have been two recent meta-analyses of the geographical distribution of α_1-antitrypsin deficiency [18,19]. The highest prevalence of the Z allele was recorded in northern and western European countries and gradually decreases towards the south east of the continent (see Figure 1). In contrast, the highest frequency of the S allele is found in southern Europe and its prevalence gradually decreases towards north-east Europe (see Figure 1). Therefore the prevalence of the Z homozygote varies from approximately 1 in

1500 in Scandinavia to approximately 1 in 2000 in the United Kingdom and on average, the frequency of the severe Z allele in all Northern Europeans approaches 4%. It is widely accepted that α_1-antitrypsin deficiency arose in Southern Scandinavia with the disease being spread to other countries whose inhabitants are of European descent. The average gene frequency in North America is on a par with the lowest end of the range reported in Europe. A survey from the St Louis, Missouri area revealed the prevalence of Z homozygotes to be approximately 1 in 2800 [20]. A 2003 study based on control cohort data on the population of North America indicated that the incidence of inheriting either an S or Z α_1-antitrypsin allele was 1 in 9.8 for Canada and 1 in 11.3 for the United States [15,19]. The gene frequency of the Z variant in Australasia is similar to that of North America [17,21]. The disease is rare in far East Asia and most cases in Japan are attributed to the Siiyama variant (Ser53Phe) rather than the Z allele [22]. The disease is also thought to be rare in South America [19] although few studies have been reported from this region. Evidence is emerging that there may be significant prevalence of the Z allele in parts of the Middle East, Central and South East Asia and the whole African continent [19] although more studies are needed.

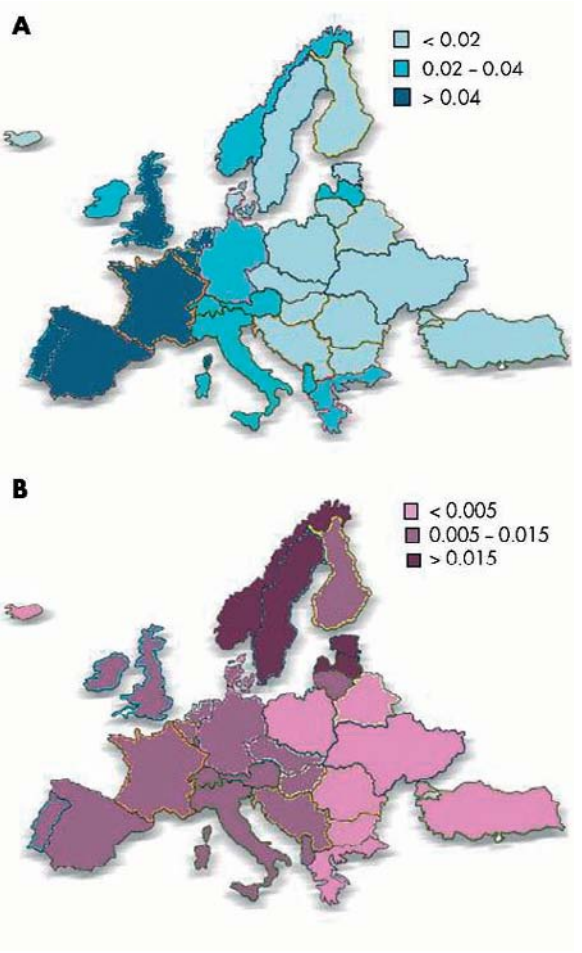

Figure 1. Frequencies of (A) PI*S and (B) PI*Z alleles in Europe. Reproduced from Luisetti and Seersholm [17] with permission.

THE STRUCTURE AND FUNCTION OF ALPHA$_1$-ANTITRYPSIN

Crystal structures have demonstrated that α_1-antitrypsin is composed of three β-sheets (A-C) and an exposed mobile reactive loop (Figure 2) that presents a peptide sequence as a pseudosubstrate for the target protease [23-27]. The critical amino acids within this loop are the P1-P1' residues, methionine-serine, as these act as a 'bait' for neutrophil elastase [28]. After docking, the enzyme cleaves the P1-P1' peptide bond of α_1-antitrypsin [29] and the protease is inactivated by a mousetrap action (Figure 2) that swings it from the upper to the lower pole of the protein in association with the insertion of the reactive loop as an extra strand (s4A) in β-sheet A [30-34]. This altered conformation of α_1-antitrypsin bound to its target enzyme is then recognized by hepatic receptors and cleared from the circulation [35-37]. The remarkable 'mousetrap' action of α_1-antitrypsin is central to its role as an effective inhibitor of serine proteases. Paradoxically, it is also its 'Achilles heel' as point mutations in these mobile domains make the molecule vulnerable to aberrant conformational transitions such as those that underlie α_1-antitrypsin deficiency.

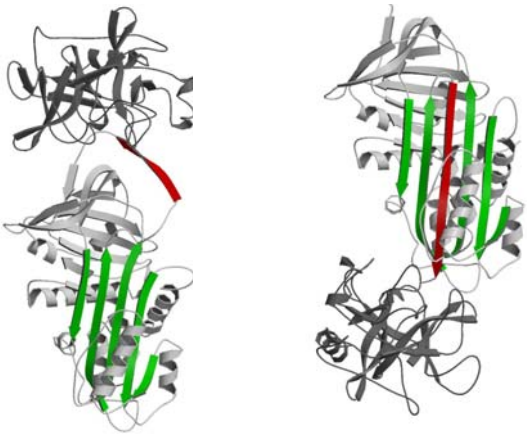

Figure 2. α_1-antitrypsin can be considered to act as a mousetrap [23,34]. Following docking (left) the neutrophil elastase (top) is inactivated by movement from the upper to the lower pole of the protein (right). This is associated with insertion of the reactive loop (red) as an extra strand into ß-sheet A (green) [38].

There is now overwhelming evidence that the liver disease associated with the Z variant of α_1-antitrypsin is due to a failure of secretion and the accumulation of aggregated protein rather than plasma deficiency. Strong support is provided by the recognition that null alleles, which produce no α_1-antitrypsin, are not associated with liver disease [39]. Moreover, the overexpression of Z α_1-antitrypsin in animal models results in liver damage [40,41]. It has been shown that the Z variant of α_1-antitrypsin is retained within hepatocytes as the mutation causes a unique conformational transition which allows a novel protein-protein interaction. The point mutation in the Z variant of α_1-antitrypsin is at residue P17 (17 residues proximal to the P1 reactive centre) at the head of strand 5 of β-sheet A and the base of the mobile reactive loop (Figure 3). The mutation opens β-sheet A, thereby favouring the insertion of the reactive loop of a second α_1-antitrypsin molecule to form a dimer [23,42-44]. This can then

extend to form polymers (Figure 3) that tangle in the endoplasmic reticulum of the hepatocyte to form the Periodic Acid Schiff (PAS) positive inclusions that are the hallmark of Z α_1-antitrypsin liver disease [42,45-47].

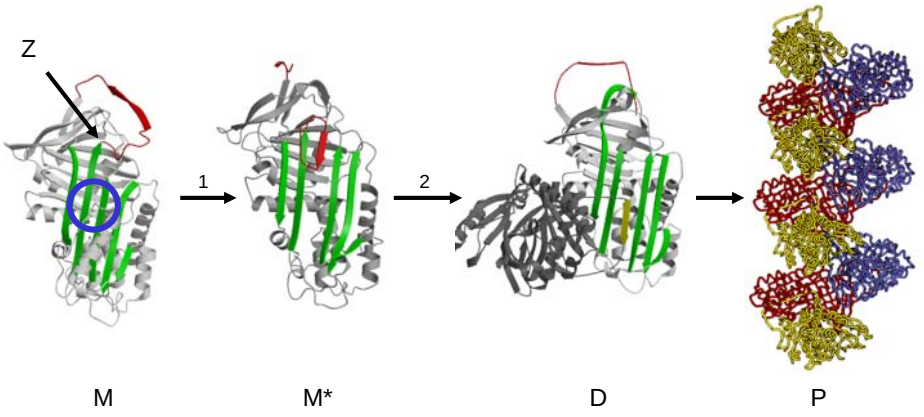

Figure 3. Pathway of serpin polymerization. The structure of α_1-antitrypsin is centered on β-sheet A (green) and the mobile reactive centre loop (red). Polymer formation results from the Z variant of α_1-antitrypsin (Glu342Lys at P_{17}; arrowed) or mutations in the shutter domain (blue circle) that open β-sheet A to favour partial loop insertion (step 1) and the formation of an unstable intermediate (M*), [43,48,49]. The patent β-sheet A can then accept the loop of another molecule (step 2) to form a dimer (D) which then extends into polymers (P).

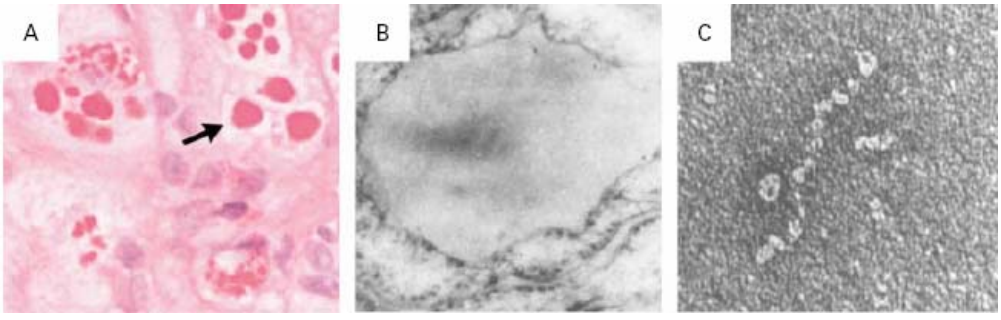

Figure 4. Z α_1-antitrypsin is retained within hepatocytes as intracellular inclusions. (A) These inclusions are PAS positive and diastase resistant (arrow) and are associated with neonatal hepatitis and hepatocellular carcinoma. (B) Electron micrograph of a hepatocyte from the liver of a patient with Z α_1-antitrypsin deficiency shows the accumulation of α_1-antitrypsin within the rough endoplasmic reticulum. These inclusions are composed of chains of α_1-antitrypsin polymers shown here from the plasma of a Siiyama α_1-antitrypsin homozygote (C). Reproduced from Carrell and Lomas [3] with permission.

Support for this hypothesis came from the demonstration that plasma purified Z α_1-antitrypsin formed chains of polymers when incubated under physiological conditions [42]. The rate of polymer formation was accelerated by raising the temperature to 41°C and could be blocked by peptides that competed for annealing to β-sheet A [42,50,51]. The role of polymerization *in vivo* was confirmed by the finding of α_1-antitrypsin polymers in inclusion bodies from the liver of Z α_1-antitrypsin homozygotes with cirrhosis [42,45,46] and in

hepatic cell lines [52] and mouse models [47] expressing the Z variant (Figure 4). Moreover, point mutations that block polymerization increased the secretion of mutants of α_1-antitrypsin from a *Xenopus* oocyte expression system [53,54].

Biochemical, biophysical and crystallographic analyses have been used to assess the pathway of α_1-antitrypsin polymerization (Figure 3) [43, 48]. Step 1 represents the conformational change of α_1-antitrypsin to a polymerogenic monomeric form (M*) and step 2 represents the formation of α_1-antitrypsin dimers and polymers (P). The presence of the unstable, polymerizing intermediate M* was predicted from the biophysical analysis of polymer formation [43] the demonstration of an unfolding intermediate [27,55,56], and solving the crystal structure of a polymerogenic mutant of α_1-antichymotrypsin [48]. The Z mutation forces α_1-antitrypsin into a conformation that approximates the unstable M* and hence favours polymer formation [49].

The accumulation of α_1-antitrypsin within hepatocytes also occurs with the two other rare mutations: Siiyama [57,58] and Mmalton [59]. These variants result from mutations in the shutter domain of α_1-antitrypsin (Figure 3).

The precise way in which α_1-antitrypsin polymers cause hepatocyte damage is still to be fully elucidated. Studies in mice transgenic for the human Z α_1-antitrypsin gene have shown that the polymers accumulate within the Endoplasmic Reticulum (ER) of hepatocytes [41,60]. These mice develop chronic liver disease and hepatocellular carcinoma despite having normal levels of circulating α_1-antitrypsin due to endogenous genes, which would imply that Z α_1-antitrypsin polymers are directly toxic to hepatocytes [61]. The quality control mechanisms within the ER of hepatocytes are currently being elucidated. It is understood that trimming of asparagine linked oligosaccharides targets Z α_1-antitrypsin polymers into a non-proteosomal disposal pathway [62] although it has been proposed that numerous proteosomal pathways are also involved in handling the polymers [63]. There is also an intense autophagic response within hepatocytes to degrade the mutant protein and it has been proposed that this results in mitochondrial damage and subsequent death of the hepatocyte [60,64,65].

The temperature and concentration dependence of polymerization may account for the wide clinical spectrum of liver disease in those patients who are homozygous for the Z allele. The synthesis of Z α_1-antitrypsin rises as part of the acute phase response and subsequent protein accumulation causes the degradative pathways to become overwhelmed thereby exacerbating hepatic injury. Recent data from a *Drosophila* model of α_1-antitrypsin deficiency shows a clear temperature dependence of polymerization *in vivo* [66]. There is also clinical evidence to suggest that high temperatures exacerbate the liver disease associated with Z α_1-antitrypsin. In a prospective study of 120 Z α_1-antitrypsin homozygotes, two patients developed progressive jaundice following episodes of systemic inflammation and many asymptomatic infants developed deranged liver function tests in association with coryzal illnesses and eczema [67,68].

THE SERPINOPATHIES

The loop sheet polymerisation that underlies Z α_1-antitrypsin associated liver disease is not restricted to α_1-antitrypsin and has now been shown to underlie the deficiency and

inactivation of other serpin variants. This common mechanism allows these disorders to be grouped together as 'the serpinopathies' [13]. Naturally occurring mutations have been described in the shutter (Figure 3) and other domains of the plasma proteins C1-inhibitor, antithrombin and α_1-antichymotrypsin. These mutations destabilize the serpin architecture to allow the formation of inactive polymers that are retained within hepatocytes. This has not been shown to cause clinically significant liver disease but does result in severe plasma deficiency, which leads to uncontrolled activation of proteolytic cascades and angio-oedema, thrombosis, and chronic obstructive pulmonary disease respectively [3,13,23,38].

The process of serpin polymerization has most recently been illustrated in the inclusion body dementia, familial encephalopathy with neuroserpin inclusion bodies (FENIB) [69]. This is an autosomal dominant dementia characterized by eosinophilic neuronal inclusions of neuroserpin. The inclusions are PAS positive and diastase resistant and bear a remarkable similarity to those formed by Z α_1-antitrypsin within the liver. The inclusions are formed of neuroserpin and affected individuals carry point mutations in the shutter domain of the protein that destabilize the protein allowing polymer formation [70].

THE CLINICAL FEATURES OF ALPHA$_1$-ANTITRYPSIN DEFICIENCY

Liver Disease

There is a broad clinical spectrum of liver disease associated with α_1-antitrypsin deficiency. Many patients remain asymptomatic throughout their lives and many others have abnormal liver function tests but no clinical sequelae [71]. It is presumed that both genetic and environmental factors alter the hepatocyte response to Z α_1-antitrypsin polymer accumulation [4,72]. There is conflicting evidence as to whether breast feeding protects against the development of chronic liver disease and early death in childhood [72,73] but there is no doubt that the accumulation of abnormal protein starts *in utero* [74] and is characterized by the accumulation of diastase-resistant, periodic acid-Schiff positive inclusions of α_1-antitrypsin in the periportal cells [75,76].

Over 70% of Z α_1-antitrypsin homozygote infants have a raised serum alanine aminotransferase in the first year of life but it only remains abnormal in 15% of children at 12 years of age [67,68,77,78]. Similarly, serum bilirubin is raised in 11% of Z homozygous infants in the first 2-4 months but usually falls to normal by 6 months of age. One in 10 infants develops cholestatic jaundice and 6% develop clinical evidence of liver disease without jaundice. Approximately 15% of these patients progress to juvenile cirrhosis. The overall risk of death from liver disease in Z homozygote children during childhood is 2-3%, with boys more at risk than girls. Z α_1-antitrypsin homozygous individuals have a 2% incidence of abnormal liver enzyme levels during adolescent years and a 5% incidence from 20-50 years of age [71]. Male gender and obesity are thought to predispose to advanced liver disease in adults with α_1-antitrypsin deficiency but there has been no proven correlation with either alcohol intake or a past history of viral hepatitis [79].

The overall incidence of decompensated liver disease is rare but all adults who are homozygous for the Z α_1-antitrypsin allele have evidence of slowly progressive hepatic damage [80,81]. This is usually subclinical and may only be evident as a minor degree of portal fibrosis without derangement of liver function tests. The presentation of patients with chronic liver disease secondary to α_1-antitrypsin deficiency is indistinguishable from that due to other causes, although typically such patients will present with asymptomatic hepatosplenomegaly and mildly abnormal liver function tests rather than with portal hypertension and its complications [71,82-84]. Baseline investigations that should be performed in all patients with suspected α_1-antitrypsin deficiency are listed in Table 2.

Table 2. Baseline investigations for patients with hepatic complications of α_1-antitrypsin deficiency.

Liver Function Tests: AST, ALT, Alkaline Phosphatase, Bilirubin, Albumin
Clotting studies: PT, APTT, Fibrinogen
Liver Ultrasound Scan
α fetoprotein
α_1-Antitrypsin levels and phenotype
Caeruloplasmin and copper levels
Viral hepatitis screen
Autoantibody screen

Necroscopic studies have shown an odds ratio of developing hepatocellular carcinoma of 5.0 in patients with Z α_1-antitrypsin deficiency, usually but not always in association with cirrhosis [4,85].

Despite there being a clear correlation between liver disease and homozygosity for the Z allele, the risk of liver disease in individuals heterozygous for the Z mutation is uncertain. It has been proposed that 'heteropolymers' consisting of the Z allele and another mutant allele, such as S or I, can form hepatic inclusions in a similar way to Z α_1-antitrypsin polymers and lead to the development of cirrhosis [16]. The S and I variants of α_1-antitrypsin have much slower rates of polymerization than the Z variant such that individuals who are homozygous for these mutations do not have clinically significant retention of polymers or plasma deficiency [16,81]. The shutter domain mutants Mmalton and Siiyama have been shown to cause both plasma deficiency and hepatic inclusions but there is currently insufficient information to state whether or not homozygotes develop progressive liver damage and cirrhosis [81].

Lung Disease

α_1-Antitrypsin deficiency is a proven genetic risk factor for chronic obstructive pulmonary disease (COPD) [86]. Smoking is the most important risk factor for the development of emphysema in Z α_1-antitrypsin homozygotes [13,87]. A lack of circulating α_1-antitrypsin leads to uncontrolled proteolytic attack from host proteinases and subsequent

tissue destruction. The mutant Z α_1-antitrypsin is also five-fold less effective at inhibiting neutrophil elastase compared to the normal M protein [50]. This results in characteristic bibasal panlobular emphysema. The inhibitory activity of Z α_1-antitrypsin can be further reduced as it is susceptible to oxidation by free radicals from leucocytes or direct oxidation by cigarette smoke [88]. The pathways underlying the development of emphysema in individuals with α_1-antitrypsin deficiency are illustrated in Figure 5.

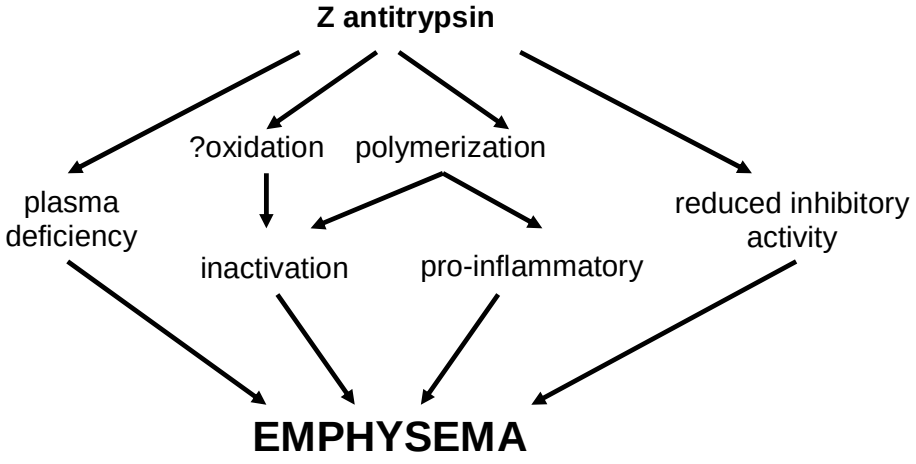

Figure 5. Model for the pathogenesis of emphysema in patients with α_1-antitrypsin deficiency. The plasma deficiency and reduced inhibitory activity of Z α_1-antitrypsin may be exacerbated by the polymerization of α_1-antitrypsin within the lungs. α_1-Antitrypsin polymers also act as a pro-inflammatory stimulus to attract and activate neutrophils. Cigarette smoke directly promotes neutrophil recruitment and creates an acidic local environment which promotes polymer formation and the oxidation and inactivation of α_1-antitrypsin. Reproduced with permission from Lomas and Mahadeva [13].

Patients with Z α_1-antitrypsin deficiency have been shown to have an excess number of neutrophils in bronchoalveolar lavage fluid and in sections of pulmonary parenchyma [13,89,90]. Studies have shown that polymers themselves are chemotactic for human neutrophils and induce neutrophil shape change, stimulate myeloperoxidase release and encourage neutrophil adhesion [91,92]. It is thought that it could be the presence of polymers that explains the progression of lung disease in Z α_1-antitrypsin homozygotes after smoking cessation, despite adequate intravenous replacement with plasma α_1-antitrypsin.

The investigations that should be carried out in a patient with pulmonary complications of α_1-antitrypsin deficiency are shown in Table 3.

Plain chest radiographs show evidence of hyperinflation, reduced lung markings and, occasionally, bulla formation. Lung function tests show evidence of obstructive airflow (with reduced FEV_1/FVC ratio), increased lung volumes, air trapping (as shown by a raised residual volume) as well as impaired gas transfer. Often symptomatic patients will be hypoxic on arterial blood gas analysis [4].

Table 3. Baseline investigations for patients with pulmonary complications of α_1-antitrypsin deficiency.

PA Chest Radiograph
Pulmonary Function Tests: Spirometry with reversibility to bronchodilators
Lung volumes and a flow volume loop
Residual volume
Diffusion capacity
Oxygen saturation ± Arterial blood gases
High Resolution CT scan
α_1-Antitrypsin levels and phenotype

OTHER MANIFESTATIONS OF ALPHA$_1$-ANTITRYPSIN DEFICIENCY

Panniculitis

Many cases of panniculitis associated with α_1-antitrypsin deficiency have been reported [4,93]. Typically individuals develop painful nodules which ulcerate, often in association with fat necrosis. Treatment options include corticosteroids, dapsone, tetracycline and intravenous α_1-antitrypsin replacement therapy. It is not known how deficiency leads to panniculitis although there are several hypotheses. These include insufficient inhibition of membrane-bound serine proteases, increased elastin degradation promoted by large amounts of fatty acids, insufficient inhibition of complement activation and neutrophil accumulation at sites of inflammation resulting in the release of serine proteases with subsequent damage to surrounding connective-tissue structures [94].

Vasculitis

α_1-Antitrypsin deficiency has been linked to systemic vasculitides, notably the cANCA (anti-proteinase 3 antibody) positive vasculitides such as Wegener's granulomatosis [4,10,11]. There is a higher prevalence of cANCA in individuals with the Z allele [12]. Proteinase 3 is a major substrate for α_1-antitrypsin and so deficiency of α_1-antitrypsin might enhance development of autoimmunity to proteinase 3. It is also possible that Z polymers may promote autoimmune vasculitic responses [12,91]. It has been recommended that all patients with cANCA positive vasculitis are tested for α_1-antitrypsin deficiency [95].

DIAGNOSIS

Serum α_1-antitrypsin levels are classically low in α_1-antitrypsin deficiency and the precise level often gives an indication as to the nature of the underlying α_1-antitrypsin variant. Normal (MM) α_1-antitrypsin is present in plasma at a concentration of 1.9-3.5 mg/ml. The S allele reduces plasma levels to 60% of normal so an MS heterozygote will have typical α_1-antitrypsin levels ranging between 1.5-2.8 mg/ml and an SS homozygote between 1.1-2.1 mg/ml. The Z allele reduces plasma levels to 10-15% of normal. Therefore an MZ heterozygote will typically have plasma levels ranging from 0.9-1.7 mg/ml, an SZ heterozygote between 0.6-1.1 mg/ml and a ZZ homozygote between 0.2-0.4 mg/ml (see Table 4).

Table 4. Serum levels of α_1-antitrypsin according to phenotype.

MM	1.9-3.5 mg/ml
MS	1.5-2.8 mg/ml
SS	1.1-2.1 mg/ml
MZ	0.9-1.7 mg/ml
SZ	0.6-1.1 mg/ml
ZZ	0.2-0.4 mg/ml

It must be remembered, however that α_1-antitrypsin is an acute phase reactant protein and serum levels will be elevated during any episodes of acute inflammation. Therefore the phenotype should always be confirmed by isoelectric focusing or by genotyping.

Naturally all patients should have their liver function tests monitored and all other causes of cirrhosis should be excluded whatever the α_1-antitrypsin phenotype or serum level, as it cannot be assumed that the chronic liver disease is solely as a result of α_1-antitrypsin deficiency.

Liver biopsy is a sensitive way of assessing hepatocyte damage by α_1-antitrypsin polymer accumulation. Histology typically reveals the characteristic diastase-resistant PAS positive globules within the hepatocyte ER when viewed either by light or electron microscopy (see Figure 4). Other typical features seen in liver biopsy specimens include mild portal fibrosis with lobular steatosis, chronic active hepatitis (featuring inflammatory infiltrate of the portal tract with piecemeal necrosis) and cirrhosis [96].

TREATMENT

Treatment strategies for the liver and lung complications of α_1-antitrypsin deficiency are highlighted in Tables 5 and 6.

Table 5. Treatment strategy for hepatic complications of α_1-antitrypsin deficiency.

Current [71,97]
Alcohol avoidance
Vitamin replacement and nutritional support and advice to ensure BMI 20-25
Vaccination against Hepatitis A and B
Supplemental fat soluble vitamins in severe disease
Paracentesis for ascites
Transjugular Intrahepatic Portosystemic Shunt (TIPS) for portal hypertension
Liver Transplantation (disease will not recur in transplanted organ)
Screening for family members
Genetic counseling
Support from community organizations USA: Alpha-1 foundation, Alpha-1 association, AlphaNet
UK: Alpha1antitrypsin alliance, Alpha1 UK
Other: Alpha 1 Canada
Future [13,98]
Strategies to prohibit polymerization
Gene Therapy
Gene Repair

Treatment of the chronic liver disease associated with α_1-antitrypsin deficiency is supportive. End stage liver disease and severe portal hypertension are indications for hepatic transplantation. α_1-Antitrypsin induced cirrhosis will not recur in the transplanted liver as the transplanted organ will produce M (normal) α_1-antitrypsin and therefore no further polymers will be formed.

Intravenous augmentation therapy with purified α_1-antitrypsin to boost low plasma levels is currently available in a few countries as a specific treatment for patients with emphysema, where it is the deficiency of the protease inhibitor that causes lung tissue destruction [98,104,105]. The goal of this treatment is to raise and maintain serum α_1-antitrypsin concentrations above the protective threshold, which is thought to be 0.8mg/ml. There have been three different preparations of human α_1-antitrypsin that have received US FDA approval for therapeutic use. The original preparation was derived from pasteurization of pooled human plasma and is called Prolastin. Prolastin is also licensed in parts of mainland Europe, South America, Canada and Ukraine [12]. More recent drugs, using solvent detergent and nanofiltration from human plasma, have subsequently been developed (Aralast, Zemaira). These newer preparations have been shown in small, randomized, double blind clinical trials to raise serum levels above the protective threshold. However these trials only compared the new drugs to prolastin with the aim of showing that their therapeutic effects were not inferior to established treatment [106,107]. To date there has been only one randomized placebo-controlled trial of augmentation therapy where patients were allocated to receive either intravenous replacement therapy or albumin infusions. Over 3 years of follow up there was no significant alteration in FEV_1 between the groups although a trend towards a slower loss of lung tissue as assessed by CT scan was noted in augmentation therapy recipients [108]. The infused protein remains active after administration and the treatment is

generally well tolerated with few important side effects recorded in studies specifically designed to address this issue [109-111]. The most common adverse events reported were dyspnoea, dizziness, syncope, chills, urticaria, nausea and fatigue. Although there are contradictory studies as to whether patients obtain a long term improvement in lung function [112-115], the 2003 international evidence based standards document of care from the American Thoracic Society and the European Respiratory Society recommends intravenous augmentation therapy in those individuals with established airflow obstruction and in those individuals who have undergone lung transplantation for emphysema associated with α_1-antitrypsin deficiency [95]. The use of intravenous augmentation therapy is, of course, of no value in treating the polymer driven liver disease.

Table 6. Treatment strategy for pulmonary complications of α_1-antitrypsin deficiency.

Current [99]
Smoking cessation (including Nicotine Replacement Therapy)
Avoidance of environmental irritants
Prevention of pulmonary infections
Influenza and Pneumonia vaccinations
Early and aggressive treatment of asthma/COPD exacerbations (bronchodilators/corticosteroids)
Early treatment of Pulmonary Hypertension and Cor Pulmonale
Regular exercise, physiotherapy, pulmonary rehabilitation
Management of anxiety and depression
Supplemental Oxygen when required as determined by arterial blood gas analysis
Intravenous α_1-antitrypsin augmentation therapy (only certain countries)
Opioids for palliative control of terminal breathlessness
Bullectomy/Lung Volume Reduction Surgery (poor outcome in α_1-antitrypsin deficiency)
Lung Transplantation
Screening for family members
Genetic counseling
Support from community organizations USA: Alpha-1 foundation, Alpha-1 association, AlphaNet
UK: Alpha1 UK, Alpha1antitrypsin alliance
Other: Alpha1 Canada
Future [98, 100-103]
Inhaled augmentation therapy
Gene Therapy

FUTURE STRATEGIES

Understanding the mechanism behind α_1-antitrypsin polymerization has allowed the development of new strategies to prevent polymerization and therefore encourage more native α_1-antitrypsin to be secreted from the hepatocytes, which would prevent hepatocyte death and increase the circulating α_1-antitrypsin concentration. Indeed it has been shown that

the polymerization of Z α_1-antitrypsin can be blocked by annealing reactive loop peptides to β-sheet A [42]. However these peptides were too long to enable rational drug design and therefore a 6-mer peptide has been produced that specifically binds to Z α_1-antitrypsin and inhibits polymerization [49]. In the future it may be possible to convert such small peptides into drugs that can be used to inhibit polymerization. More recently a hydrophobic pocket has been identified in α_1-antitrypsin that is bounded by strand 2A and helices D and E [26,116]. This cavity is patent in the native protein but is filled during the polymerization process when β-sheet A accepts an endogenous reactive loop peptide [26]. Introducing mutations into this pocket retards polymerization and increases the secretion of Z α_1-antitrypsin from a *Xenopus* oocyte expression system [54]. This cavity is therefore an ideal target for the development of drugs that will stabilize β-sheet A and therefore prevent polymerization. A range of compounds that are suspected to perform such a task have been selected by computational analysis and are currently being screened *in vitro*.

Another strategy involves the use of chemical chaperones to stabilize intermediates on the folding pathway. Osmolytes such as betaline, trimethylamine oxide and sarcosine all stabilize α_1-antitrypsin against polymer formation [117]. Glycerol has been shown to bind to and stabilize β-sheet A and increase Z α_1-antitrypsin secretion from cell lines [118,119]. Similarly 4-phenylbutyrate (4-PBA) increases expression of Z α_1-antitrypsin from cell lines [118] and has been shown to increase the expression of mutant (ΔF 508) cystic fibrosis transmembrane regulator protein both *in vitro* and *in vivo* [120,121]. A pilot study is currently being carried out to evaluate the potential of 4-PBA to promote the secretion of α_1-antitrypsin in patients with α_1-antitrypsin deficiency, although preliminary results have not been encouraging [122].

Gene therapy trials have largely been directed at treating the respiratory complications of α_1-antitrypsin deficiency. This is because the introduction of a normal gene does not reduce the production of the endogenous abnormal gene product. Therefore hepatocyte damage will occur regardless of the total serum α_1-antitrypsin concentration. Several studies have suggested that an adeno-associated virus (AAV) mediated delivery of α_1-antitrypsin is a potential strategy for successful gene therapy [100-103]. A recent study has reported that intrapleural administration of an AAV5 vector may provide a potential therapeutic route [123].

In order for gene therapy to prevent the liver complications of α_1-antitrypsin deficiency any potential therapy would need to inhibit the expression of the mutated gene and replace it with a normally functioning one. This has been achieved *in vitro* using site specific ribozymes to cleave the α_1-antitrypsin mRNA at a specific site to prevent abnormal protein production, followed by subsequent retroviral transduction of a normal gene into the same cell line [124]. *In vivo* work in this field has involved the use of transgenic mice carrying the human Z α_1-antitrypsin allele. The mice were treated via an indwelling portal vein catheter with a simian virus 40 (SV40) derived vector carrying a ribozyme designed to target the human transcript. This resulted in significant reduction in production of human Z α_1-antitrypsin and therefore reduced accumulation of the abnormal protein. Moreover when normal mice were treated with an SV40-derived vector containing normal human α_1-antitrypsin that was resistant to ribozymal cleavage, high levels of human α_1-antitrypsin were expressed [125].

Other, more speculative, future approaches include a potential role for gene repair. Chimeric RNA/DNA oligonucleotides have been used in model systems to amend a single gene mutation [98,126]. More recently encouraging results have been obtained with single stranded bare DNA oligonucleotides both *in vivo* and *in vitro* [98,127]. There may also be a potential role for stem cell therapy in α_1-antitrypsin deficiency although this needs further evaluation [128].

PROGNOSIS

According to the Death Review Committee (DRC) of the National Heart, Lung and Blood Institute Registry, individuals with severe α_1-antitrypsin deficiency have an excess mortality linked to lung and liver disease. In the subject population (studied over 7 years in 37 centers across North America) emphysema accounted for 85% of mortality and cirrhosis a further 12% [129]. Liver failure accounted for 25% of deaths in those patients who had never smoked.

CONCLUSION

α_1-Antitrypsin deficiency is a well recognized cause of both emphysema and chronic liver failure. In the years to come it is to be hoped that there will be an increasing awareness of α_1-antitrypsin deficiency and this should lead to the condition being diagnosed at younger ages. Therefore, the incidence of lung disease should be significantly reduced as patients can be advised about the dangers of cigarette smoking and other family members can be screened. Tackling the liver disease associated with α_1-antitrypsin deficiency represents more of a clinical conundrum and will most probably rely on an efficient screening programme, coupled with either genetic manipulation or the prevention of Z α_1-antitrypsin polymerization.

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Chapter X

GLYCOGEN STORAGE DISEASES

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ABSTRACT

The glycogen storage diseases (GSDs) or glycogenoses comprise several inherited diseases caused by abnormalities of the enzymes that regulate the synthesis or degradation of glycogen. Advances in molecular genetics [1,2] have led to the identification of the precise genetic abnormalities that cause the specific impairments of enzyme function of the various GSDs. Likewise, improved understanding of the pathophysiologic derangements resulting from individual enzyme defects has led to the development of effective nutritional therapies for these disorders [3,4]. For example, in type I GSD (GSD I), a disease that formerly was characterized by severe growth failure and delayed puberty, meticulous adherence to dietary therapy prevents hypoglycemia, ameliorates the biochemical abnormalities, decreases the size of the liver, and results in normal or nearly normal physical growth and development. Long-term complications, including nephropathy that can progress to renal failure, and hepatic adenomata that can hemorrhage or become malignant and may be associated with severe anemia, are a major concern in GSD I. In type III GSD (GSD III), the liver decreases in size during puberty; however, adults uncommonly develop cirrhosis, and patients with absent muscle glycogen debrancher enzyme activity develop progressive debilitating myopathy and cardiomyopathy. It is unclear whether these complications can be prevented by nutritional therapy. The severe form of type IV GSD (GSD IV) rapidly progresses to cirrhosis with portal hypertension and liver failure and no specific treatment, other than a liver transplant, is currently available. GSDs caused by lack of phosphorylase activity are milder disorders with a good prognosis.

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INTRODUCTION

The glycogen storage diseases (GSDs) or glycogenoses comprise several inherited diseases caused by abnormalities of the enzymes that regulate the synthesis or degradation of glycogen (Figures 1) [3,5]. Glycogen is a highly branched polymer of glucose and is the storage form of glucose in mammals. The major sites of glycogen deposition are skeletal muscle and liver, but many cell types are capable of glycogen synthesis, including cardiac and smooth muscle, the kidney, brain and even adipose tissue. Glycogen comprises approximately 4-6 percent and 1-2 percent of the wet weight of the liver and skeletal muscle, respectively. In the average well-fed man consuming a diet rich in carbohydrate about 80 g of glycogen is stored in the liver and 400 g in skeletal muscle [6].

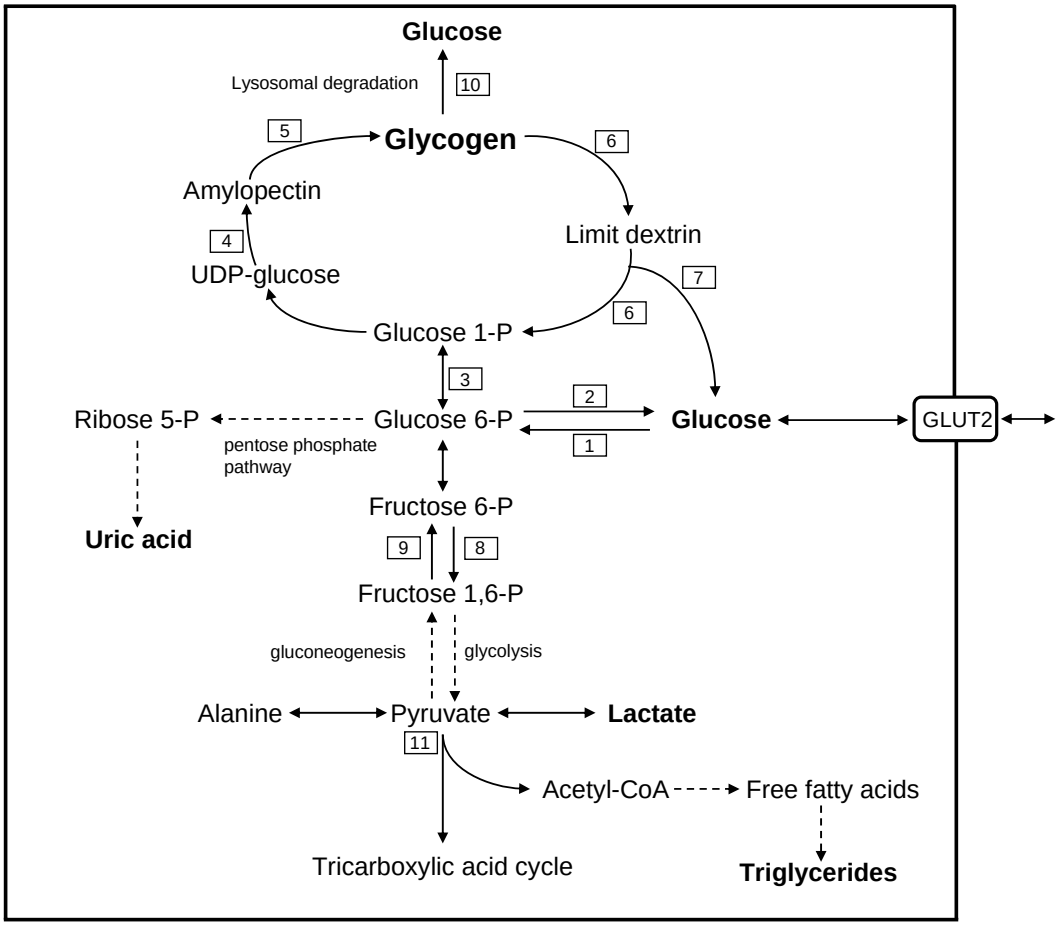

Figure 1. Simplified scheme of glycogen synthesis and degradation in the liver. Note that in skeletal muscle GLUT-4, transports glucose across the cell membrane and glucose-6-phosphatase is absent. UDP-glucose is uridine diphosphoglucose; 1. hexokinase/glucokinase, 2. glucose 6-phosphatase, 3. phosphoglucomutase, 4. glycogen synthase, 5. branching enzyme, 6. glycogen phosphorylase, 7. debranching enzyme, 8. phosphofructokinase, 9. fructose 1,6-bisphosphatase, 10. acid maltase, 11. pyruvate dehydrogenase.

Glucose transporter-2 (GLUT2) is the most important facilitative glucose transporter in hepatocytes, pancreatic ß-cells, and the basal membranes of renal proximal tubular cells and intestinal mucosal cells [7]. It has a high K_m (~ 40mmol/L); consequently, the free glucose concentration in hepatocytes increases in direct proportion to the increase in plasma glucose concentration. After a meal, exogenous glucose delivery increases at rates largely determined by the carbohydrate content of the ingested food and the rate of gastric emptying. Endogenous glucose production is suppressed, and excess glucose is either metabolized or stored as glycogen in skeletal muscle and the liver [8].

Glycogen synthesis and degradation in the liver follow distinct pathways that begin and end with glucose-1-phosphate (G1P) (Figure 1) [9]. The liver is freely permeable to glucose, which is rapidly phosphorylated by glucokinase to form glucose-6-phosphate (G6P) before it can enter one of several metabolic pathways. It can be reversibly converted to G1P, the starting point for glycogen synthesis (Figure 1). G1P reacts with uridine triphosphate to form uridine diphosphate (UDP)-glucose. Glycogen synthase catalyzes the formation of α-1,4-linkages from UDP-glucose, which elongates chains of glucose molecules. A branching enzyme forms the α-1,6-linkages at branch points along the chain making glycogen a branched polymer. Alternatively, G6P can be hydrolyzed to glucose by glucose-6-phosphatase or it can be metabolized via the glycolytic pathway to pyruvate and lactate or via the pentose phosphate pathway to ribose-5-phosphate, a precursor of nucleotide synthesis. A cascade of enzymatic reactions activates hepatic glycogen phosphorylase, the rate-limiting enzyme of glycogenolysis, which removes glucose from the outer branches of glycogen, yielding G1P (Figure 2).

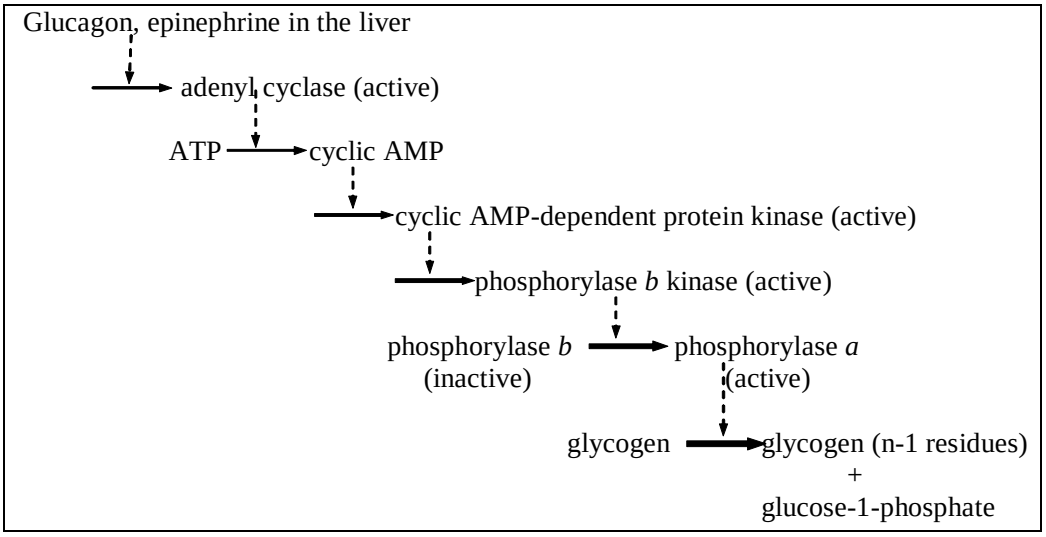

Figure 2. The glycogenolysis cascade. Phosphorylase *b* kinase also catalyzes the conversion of glycogen synthase from a more to a less active form (not shown). Because of these reciprocal changes, glycogen degradation is active when glycogen synthesis is inactive, and vice versa.

The GSDs are all inherited in an autosomal recessive manner, with the exception of type IX (which has both autosomal and X-linked inheritance), and are caused by mutations in the genes that code for enzymes involved in the synthesis or degradation of glycogen in liver

and/or muscle (Table 1). The overall frequency of GSD is approximately 1 case per 20,000-25,000 births. They are characterized by an abnormal tissue concentration and/or abnormal structure of the glycogen molecule. The GSDs may involve skeletal and cardiac muscle and liver and are referred to either by the deficient enzyme or by a number that reflects the historical sequence of their description. Twelve distinct types of GSD have been identified. They are all uncommon and some are extremely rare. Seven types of GSD account for about 97 percent of cases [5]. Those that predominantly involve the liver will be discussed in this chapter: GSD 0 (≤1%), GSD I (25%), GSD III (24%), GSD IV (3%), GSD VI and IX[1] (30%), and Fanconi-Bickel syndrome (<1%).

Hypoglycemia is the primary manifestation of the hepatic glycogenoses, whereas weakness and muscle cramps are the predominant features of the muscle glycogenoses. The hepatic glycogen storage diseases, with the notable exception of GSD IV, are characteristically associated with hypoglycemia (Table 1).

Table 1. Hepatic Glycogen Storage Diseases.

Disorder	Affected tissue	Enzyme	Inheritance	Gene	Chromosome
Type 0 GSD	Liver	Glycogen synthase	AR*	GYS2	12p12.2
Type Ia GSD	Liver, kidney, intestine	Glucose-6-phosphatase	AR	G6PC	17q21
Type Ib GSD	Liver	Glucose-6-phosphate transporter	AR	G6PT1	11q23
Type IIIa GSD	Liver, muscle, heart	Glycogen debranching enzyme (GDE)	AR	AGL	1p21
Type IIIb GSD	Liver	Glycogen debranching enzyme	AR	AGL	1p21
Type IV GSD	Liver, muscle, heart	Glycogen branching enzyme	AR	GBE1	3p12.3
Type VI GSD	Liver	Glycogen phosphorylase	AR	PYGL	14q21-22
Type IX GSD†	Liver, erythrocytes, leukocytes	Liver isoform of α subunit of phosphorylase kinase	X-linked	PHKA2	Xp22.2 p22.1
	Liver, muscle, erythrocytes, leukocytes	ß subunit of liver and muscle phosphorylase kinase	AR	PHKB	16q12-q13
	Liver	Testis/liver isoform of γ subunit of phosphorylase kinase	AR	PHKG2	16p11-p12
Fanconi-Bickel syndrome (Type XI GSD)	Liver, kidney, pancreatic ß cells, intestine	Glucose transporter 2**	AR	GLUT2	3q26.1-q26.3

*AR autosomal recessive; sometimes designated Type VIII:

**GLUT2 is a facilitative glucose transporter, not an enzyme.

[1] Also designated as GSD type VIII

GLYCOGEN SYNTHASE DEFICIENCY (TYPE 0 GLYCOGEN STORAGE DISEASE, GSD 0)

Type 0 glycogen storage disease is caused by mutations in the GYS2 gene which result in deficiency of the hepatic isoform of glycogen synthase [10]. To date, 15 different mutations have been documented. The only common mutation is in exon 4 (R246X) and has been found in patients of Italian descent both in Europe and in North America. Cases of GSD0 have been identified throughout Europe, North and South America. GSD0 has a classic autosomal recessive inheritance.

Clinical Features

Although this disorder has been classified as a GSD, this is really a misnomer because, in contrast to all other types of glycogenoses, which are characterized by increased tissue glycogen content, deficiency of glycogen synthase causes a marked decrease in liver glycogen content. GSD 0 is the only hepatic GSD *not* associated with hepatomegaly.

Because a substantial fraction of dietary carbohydrate is normally stored in the liver as glycogen, inability to synthesize hepatic glycogen causes postprandial hyperglycemia after ingestion of a carbohydrate-containing meal. Glucose and other dietary sugars taken up by the liver are shunted into the glycolytic pathway (Figure 1) leading to postprandial hyperglycemia, hyperlacticacidemia, and hyperlipidemia [11]. Ketotic hypoglycemia develops with fasting [12,13]. Intact gluconeogenesis and fatty acid oxidation blunt the decrease in blood glucose levels in the postabsorptive period and explains why hypoglycemia is typically milder in this disorder than in some of the other hepatic glycogenoses. When fasting is more prolonged, however, severe hyperketonemia and hyperfattyacidemia inhibit release of alanine from skeletal muscle [14,15] leading to a reduction in precursors for gluconeogenesis and more severe hypoglycemia. Thus, the classical biochemical phenotype is alternating mild postprandial hyperglycemia and hyperlacticacidemia with fasting hypoglycemia and hyperketonemia ("ketotic hypoglycemia") [11,16].

Children with GSD 0 are usually asymptomatic during infancy, but weaning from overnight feeding often proves difficult and, when overnight feeding is stopped, fasting ketotic hypoglycemia and irritability or lethargy before breakfast is common. Despite hypoglycemia, patients may be relatively asymptomatic because hyperketonemia provides the brain with an alternative fuel [17]. Patients may be asymptomatic unless they are ill [13]. Postprandial hyperglycemia and glucosuria may be mistaken for early diabetes or renal glucosuria [16]. Most children with GSD 0 are identified incidentally when hypoglycemia is discovered during an evaluation for lethargy associated with a gastrointestinal illness or other cause of poor dietary intake. The manifestations of GSD 0 are frequently subtle and children may first come to medical attention because of short stature, failure to thrive, hyperlipidemia, or elevated hepatic transaminase levels [13].

Short stature and osteopenia are common in untreated children, but improve with prevention of hypoglycemia, lactic acidosis, and ketosis. The long-term complications commonly seen in the other forms of glycogen storage disease, such as hepatic adenomas,

cirrhosis, kidney dysfunction, and muscular abnormalities, have not been reported in adolescents or adults with GSD 0. There are few reports of adults with GSD 0, and the oldest case documented in the literature is 34 years of age [10]. All of the adults with GSD 0 have done well and there is reason to believe that the prognosis is excellent despite the lack of reported older individuals. A 26-year old woman with GSD 0 gave birth to a healthy term infant, but overnight hypoglycemia and ketonemia developed when supplemental carbohydrate was not provided in the 2nd and 3rd trimesters of pregnancy [18].

Diagnosis

Home blood glucose and urine ketone monitoring, initially, maybe used to screen for this disorder because fasting hypoglycemia and ketonuria are universal in children less than 5 years of age. If fasting ketotic hypoglycemia is demonstrated, frequent measurements of blood glucose, lactate, and ketones in both the fed (or after an oral glucose tolerance test) and fasting states (24-hour metabolic profile) show the pathognomonic biochemical disturbances [11,16]. It is important to note that a "typical" fasting study, which does not measure blood metabolite concentrations in the postprandial period, may show no obvious hormonal or biochemical abnormalities, leading to a misdiagnosis of "ketotic hypoglycemia" or "accelerated starvation" [16]. Despite the decrease in hepatic glycogen content, the glycemic response to glucagon is variable and, for poorly understood reasons, may even be near-normal [12,19]. A glycemic response to glucagon does not rule out the disorder.

In the past, the definitive diagnosis depended on performing a liver biopsy. Hepatocytes contain small amounts of glycogen and show moderate steatosis. The glycogen content is low (~0.5%; normally 1-6% wet liver weight), but not completely absent, suggesting residual hepatic glycogen synthase activity or the existence of an alternative pathway for glycogen synthesis. The diagnosis can now be confirmed non-invasively by mutational analysis of the GYS2 gene using DNA extracted from blood or saliva [10,13]. A few cases of biopsy proven GSD 0 have been diagnosed in whom no mutations could be found in GYS2.

Management

The goal of treatment is to prevent hypoglycemia and to minimize systemic acidosis by preventing postprandial hyperlacticacidemia and fasting hyperketonemia [20]. Fasting hypoglycemia, especially in young children, is prevented by a bedtime feeding of uncooked cornstarch (1-1.5 gram per kg) in low fat or skim milk. During the day, patients are fed frequently (e.g., every four hours) and the diet should contain an increased amount of protein to provide substrate for gluconeogenesis and proportionately less carbohydrate (complex starches with a low glycemic index) to minimize postprandial hyperglycemia and hyperlacticacidemia [13]. Exertional fatigue is common in some individuals, and glucose and protein supplementation often improves stamina during sports and other periods of physical activity.

GLUCOSE 6-PHOSPHATASE DEFICIENCY (TYPE 1 GLYCOGEN STORAGE DISEASE [GSD I]; VON GIERKE DISEASE; HEPATORENAL GLYCOGENOSIS)

Glucose 6-phosphatase catalyzes the terminal reaction of glycogenolysis and gluconeogenesis, the hydrolysis of G6P to glucose and inorganic phosphate in hepatocytes and renal epithelial cells (Figure 1) [21]. G6Pase is a multicomponent enzyme system located in the endoplasmic reticulum (ER) membrane and consists of nine transmembrane spanning domains. The active site faces into the ER lumen [22]. Three proteins transport the substrate, G6P, and the products, phosphate, inorganic orthophosphate, and glucose across the ER membrane. G6P transporter transports G6P into the ER. Glucose is transported out of the ER by GLUT2 [21].

More than 85% of patients with GSD 1 have deficient catalytic activity of the G6Pase system, which causes type Ia GSD (GSD Ia). More than 80 different mutations have been found in the gene (G6PC located on chromosome 17q21) that encodes G6Pase in patients with GSD Ia. The common mutations in GSD Ia are shown in Table 3. The incidence of this disorder is estimated to be 1 in 100,000 births. GSD Ia occurs in all ethnic groups. Common mutations have been found in the Ashkenazi Jewish [23], Chinese, Japanese, and Mexican populations. These mutations have not been found in patients with type Ib GSD (GSD Ib), which is caused by failure to transport G6P into the lumen of the ER owing to a mutation in the gene (G6PT1) that causes deficiency of the G6P transporter [24]. To date, approximately 65 mutations in the G6PT gene have been described. Most mutations are in exon 8; sequencing of this exon detects 75% of mutant alleles.

Table 2. Biochemical Characteristics of the Hepatic Glycogen Storage Diseases.

Type	At time of hypoglycemia			Response to oral glucose		Response to glucagon 4-8 h after meal*		Response to glucagon 2 h after meal	
	Triglyceride	Uric acid	Lactate	Glucose	Lactate	Glucose	Lactate	Glucose	Lactate
GSD-0	N	N	N	↑↑	↑↑	0-↑	0	↑	↓
GSD-I	↑↑↑	↑↑	↑↑↑	↑	↓↓	0	↑↑↑	0	↑↑
GSD-III	↑	N	N	↑	↑	0	0	↑	0
GSD-VI, IX	0-↑	N	N	↑	↑	0-↑	0	↑	0

*after meal containing carbohydrate; subjects with suspected GSD-I should not be permitted to fast for more than 4 hours; N normal, 0 no increase, 0-↑ variable increase, ↑ mild increase, ↑↑ moderate increase, ↑↑↑ marked increase, ↓ mild decrease, ↓↓ moderate decrease.

Clinical Features

GSD I is characterized by impaired production of glucose from glycogenolysis and gluconeogenesis resulting in severe hypoglycemia and increased production of lactic acid, triglyceride, and uric acid (Figure 1, Table 1). Symptoms of hypoglycemia typically occur

when the infant starts to sleep through the night (usually at 3-6 months of age) or when intercurrent illness disrupts normal feeding. The disorder may be discovered when the child presents with tachypnea, seizures, lethargy, or developmental delay. Untreated patients may have a cushingoid appearance, failure to thrive, a markedly enlarged liver, and protuberant abdomen. Social and cognitive development usually is not affected unless the infant suffers cerebral damage from recurrent hypoglycemic seizures [25].

Table 3. Common mutations in GSD Ia.

Mutation	Base change	Location of mutation	Population
R83C	C326T	Exon 2	Ashkenazi Jewish Eastern European
R83H	G327A	Exon 2	Chinese
130X	459insTA	Exon 3	Mexican Central American
212X	G727T	Exon 5	Japanese
Q347X	C1118T	Exon 5	Western European

During infancy, the blood glucose concentration decreases to less than 45 mg/dL (2.5mmol/L) within two to three hours of a feed. Ketogenesis is impaired despite hyperfattyacidemia [26]. Longer intervals between feeds cause even more severe hypoglycemia accompanied by pronounced hyperlacticacidemia and metabolic acidosis. Adaptation to hypoglycemia can occur in untreated or inadequately treated patients because hyperlactatemia provides an alternative substrate for cerebral fuel metabolism [27]. Serum uric acid is increased and liver transaminases are usually mildly elevated. The serum of untreated patients may be cloudy or milky with very high triglyceride concentrations and moderately increased levels of phospholipids, total and LDL-cholesterol, whereas the HDL-cholesterol concentration is low [28,29]. Severe hypertriglyceridemia may lead to eruptive xanthomata on the extensor surfaces of the extremities and buttocks and is associated with an increased risk of acute pancreatitis [30,31]. Paradoxically, despite their atherogenic lipid and lipoprotein profiles, the risk of cardiovascular disease does not appear to be increased [32,33]. A bleeding tendency is caused by impaired platelet function, which is secondary to the systemic metabolic abnormalities and is correctable by improving the metabolic state [34]. The numerous biochemical and hematological abnormalities observed in GSD I are summarized in Table 4.

Patients with GSD Ib have similar symptoms with the addition of neutropenia and inflammatory bowel disease. The neutropenia is a consequence of disturbed myeloid maturation, and can be either cyclical or chronic. Its severity ranges from mild to complete agranulocytosis. Neutropenia is accompanied by functional defects of circulating neutrophils and monocytes and is associated with recurrent bacterial infections. Rare cases of atypical GSD Ib without neutropenia or recurrent bacterial infections may be caused by distinct mutations that leave some residual G6P transporter activity [35]. The GSD Ib phenotype (neutropenia, neutrophil dysfunction and recurrent infections) has recently been described in patients with GSD Ia who have homozygous G188R mutations of the G6Pase gene, but no identifiable mutations in the G6P transporter gene [36]. In a recent European Study, neutropenia was documented before the age of one year in two-thirds of patients, but in 18%

of patients was first noted between the ages of six and nine years. Most patients had intermittent neutropenia without any clear cyclical course [37]. Children with GSD Ib are prone to oral complications, including recurrent mucosal ulceration, gingivitis, and rapidly progressive periodontal disease. Therapy with recombinant human granulocyte colony stimulating factor (GCSF) improves infection-related morbidity by increasing numbers of circulating neutrophils and improving *in vitro* neutrophil function [38]. Patients with GSD Ib almost universally develop a Crohn's-like inflammatory bowel disease (IBD) [39]. While the IBD responds to therapy with GCSF [37,40], this comorbidity continues to occur even when neutropenia is treated. Periodic screening of inflammatory markers is recommended. Colonoscopy should be performed when clinical and laboratory features suggest he presence of IBD. The IBD in GSD Ib may be isolated to the small intestine; consequently, a capsule endoscopy may reveal disease in patients in whom a colonoscopy reveals no evidence of bowel inflammation.

Table 4. Laboratory Abnormalities in Untreated Patients with Type I GSD.

- Hypoglycemia
- Hyperlacticacidemia
- Hyperfattyacidemia
  - mild hyperketonemia
- Metabolic acidosis with increased anion gap
- Hepatic transaminase (aspartate aminotransferase [AST], alanine aminotransferase [ALT]) levels increased
- Hyperlipidemia
  - increased total and LDL-cholesterol
  - increased phospholipids
  - markedly increased triglycerides
  - decreased HDL-cholesterol
- Hyperuricemia
- Hypercalcemia
- Inflammatory markers (erythrocyte sedimentation rate and C-reactive protein) elevated
- Anemia
- Thrombocytosis
- Neutropenia (cyclic or constant)*
- Prolonged bleeding time
  - decreased platelet adhesiveness
  - abnormal platelet aggregation
  - impaired ADP release in response to collagen and epinephrine
- Increased glomerular filtration rate
- Proximal renal tubular dysfunction
  - glucosuria
  - phosphaturia
  - generalized aminoaciduria
- Distal renal tubular dysfunction
  - acidification defect
  - hypercalciuria
  - hypocitraturia

*GSDIb

Proximal tubular dysfunction (glucosuria, phosphaturia, hypokalemia, and generalized aminoaciduria) is reversible with improved biochemical control of the disease [41]. Treated children usually show no significant impairment of renal function except glomerular hyperfiltration. Some patients have a distal renal tubular acidification defect associated with hypercalciuria [42]. Urinary citrate excretion normally increases with age, whereas in GSD Ia, there is an inverse relationship between age and citrate excretion [43]. The combination of low citrate excretion and hypercalciuria appears to be important in the pathogenesis of nephrocalcinosis and nephrolithiasis. Citrate supplementation may prevent or ameliorate nephrocalcinosis and the development of urinary calculi [43]. Increased albuminuria may be observed in adolescents. More severe renal injury with proteinuria, hypertension, and decreased creatinine clearance due to focal segmental glomerulosclerosis and interstitial fibrosis, which ultimately progresses to renal failure, may be seen in young adults [44,45]. Patients with persistently elevated concentrations of blood lactate, lipids and uric acid appear to be at increased risk of nephropathy [46]. Normalization of metabolic parameters decreases proteinuria, and optimal therapy from an early age may delay or prevent renal disease [46,47].

Hepatic adenomas are detectable in the majority of patients by the time they are adults [48]. They are usually first observed in the second and third decades of life, but may appear before puberty. Adenomas may undergo malignant degeneration or hemorrhage and are frequently associated with chronic iron resistant anemia [49]. This form of iron resistant anemia has been associated with large hepatic adenomas (>7 cm in diameter); hepcidin, which inhibits intestinal absorption of iron and macrophage recycling of iron, is inappropriately expressed in these adenomas. Resection of the hepatic adenoma(s) results in rapid correction of the anemia [47]. Ultrasonography is the preferred method of screening for hepatic adenomas, which appear as focal lesions. Magnetic resonance imaging provides greater definition when malignancy is suspected because of a worrisome change in sonographic appearance [50]. Serum α-fetoprotein levels are normal in patients with adenomas, but have been increased in some cases of hepatocellular carcinoma. Serum α-fetoprotein is not sensitive for diagnosing hepatocellular carcinoma. A recent case series found normal concentrations early in the disease in 6 of 8 patients [50]. In our experience, continuous glucose therapy from infancy does not prevent the development of focal hepatic lesions and there is no difference in the rate of adenoma formation in children treated with cornstarch compared with those treated with continuous overnight feeds [49].

With patients surviving into adulthood, osteoporosis has emerged as an important cause of morbidity. Osteoporosis develops without abnormalities in calcium, phosphate, parathyroid, or vitamin D metabolism. Poor metabolic control is associated with decreased bone mineral content, but the etiology is multifactoral, including systemic acidosis, elevated cortisol concentrations, delayed pubertal development, inadequate dietary calcium, low vitamin D concentrations, and lack of physical exercise [51,52].

Diagnosis

GSD Ia and Ib are usually suspected on the basis of their characteristic clinical and biochemical abnormalities (Table 2) and now usually can be confirmed by mutation analysis, eliminating the need to perform a liver biopsy and enzyme assay [53].

Management

Treatment consists of providing a continuous dietary source of glucose to prevent blood glucose from falling below the threshold for glucose counterregulation, approximately 70mg/dL (4mmol/L) [3]. A continuous source of glucose can be provided by nocturnal intragastric infusion (via nasogastric tube or gastrostomy) or by using uncooked (raw) cornstarch. An estimate of the minimum amount of glucose required can be obtained by using the formula to calculate the basal glucose production rate:

$$y = 0.0014x3 - 0.214x^2 + 10.411x - 9.084,$$

where y = mg glucose per minute, and x = body weight in kg [54]. Modification of the amount and/or schedule of glucose is based on the results of clinical and biochemical monitoring. In infants, we recommend 2-3 hourly feedings of a non-lactose containing formula during the day and 3 hourly feedings at night to provide an amount of glucose that equals or exceeds the calculated glucose production rate. If nighttime feedings are problematic, continuous feedings of the same formula should be given with an infusion pump.

Uncooked cornstarch acts as an intestinal reservoir of glucose that is slowly absorbed into the circulation. In many centers, cornstarch has replaced frequent daytime feedings of glucose (or glucose polymers) and continuous nocturnal intragastric glucose infusion. It can be gradually introduced at 6-12 months of age as an alternative method of glucose delivery [55]. The advantage of cornstarch is that it allows feeds to be more widely spaced, minimizes plasma glucose fluctuations and, because blood glucose levels tend to decline more slowly, blood lactate concentrations increase sufficiently to provide the brain with an alternative fuel. This decreases the risk of hypoglycemia-induced seizures. In older children, adolescents, and in adults, cornstarch is given in a slurry of water or artificially sweetened fluid at 3-5 hour intervals during the day and at 4-6 hour intervals overnight. The optimum feeding schedule and amounts of cornstarch for patients of different ages is determined by metabolic monitoring to ensure that the biochemical goals of therapy are achieved, viz., normal blood glucose levels and blood lactate concentrations $\leq$2.2 mmol/L [3,56,57]. The requirement for nocturnal glucose therapy is lifelong [58].

When hypoglycemia and hyperlacticacidemia are prevented, liver size decreases, growth improves, and serum uric acid, cholesterol and triglyceride concentrations are restored to near normal. If severe hyperuricemia persists, allopurinol should be used to lower uric acid to normal levels. Lipid-lowering agents (e.g., gemfibrozil) are seldom required, but are

indicated in patients when, despite optimal glucose therapy, persistent severe hyperlipidemia poses a significant risk of acute pancreatitis.

Dietary fat should be restricted to about 20% of the total energy intake, equally distributed among monounsaturated, polyunsaturated, and saturated fats and cholesterol is restricted to <300 mg/day. Foods that contain fructose and galactose must be restricted. Carbohydrates, mostly in the form of starches, typically provide about 60-65% of the daily calories, of which cornstarch accounts for 30 to 45%. With glucose requirements prescribed, the total caloric intake is determined largely by the child's appetite as long as the rate of weight gain is not excessive, taking into account that the diet must provide adequate amounts of protein, fat, minerals, and vitamins to support normal growth. Patients treated intensively from infancy attain adult heights within one standard deviation score of their target heights, but mild to moderate obesity is common [49,59].

AMYLO-1,6-GLUCOSIDASE DEFICIENCY (TYPE III GLYCOGEN STORAGE DISEASE; GLYCOGEN DEBRANCHING ENZYME [GDE] DEFICIENCY; LIMIT DEXTRINOSIS; CORI DISEASE; FORBES DISEASE)

Release of glucose from glycogen stores requires the combined actions of glycogen phosphorylase and GDE, which consists of two independent catalytic activities on a single polypeptide chain, an oligo-1,4→1,4 glucan transferase and amylo-1,6-glucosidase. The two activities are determined at separate catalytic sites on the polypeptide chain and can function independently of each other. After phosphorylase has acted exhaustively on the outer branches of glycogen, four glucosyl residues remain distal to the branch point (limit dextrin). Transferase activity transfers three glucose residues from one short outer branch to the end of another thus exposing the branch-point (an α-1,6-linkage). Glucosidase then hydrolyzes the branch-point permitting phosphorylase access to the α-1,4-linkages. The transferred dextrin may be further depolymerized by phosphorylase. Full debranching enzyme activity requires both the transferase and glucosidase activities. In the absence of debrancher activity, breakdown of glycogen is arrested when the outermost branch points are reached. Only 1,4 segments distal to the outermost branch points are accessible to phosphorylase and can yield glucose. This results in accumulation of an abnormal form of glycogen, phosphorylase limit dextrin, in affected tissues.

A single gene (AGL) located at 1p21 with 35 exons encodes GDE in liver and muscle [60,61]. Differential RNA transcription results in the generation of muscle and liver isoforms, with different tissue-specific promoters and an alternative usage of the first exon. At least six transcript isoforms are produced by alternative splicing with different tissue distributions [60]. Both type IIIa GSD (liver and muscle) and type IIIb (liver only) have mutations in the same gene [62]. The incidence of GSD III is estimated to be 1 in 100,000 live births. The highest prevalence of GSD IIII, due to the R408X mutation, is in the Faroe Islands, [63]. There is an increased prevalence in the Inuit population in Canada [64]. In Israel the disease is common (1 in 5,400) in Sephardic Jews of North African origin who have a common

mutation (4,455delT) that causes deficient GDE activity in both liver and muscle [65]. In the U.S.A. 80-85% of patients have type IIIa. Selective loss of one of the two GDE activities, glucosidase (type IIIc) or transferase (type IIId), is rare.

Clinical Features

Clinical and enzymatic variability is a feature of GDE deficiency [66,67]. The disease may be indistinguishable from GSD-I during infancy and early childhood. Hepatomegaly, fasting hypoglycemia with ketosis, and hyperlipidemia are the predominant features. Serum transaminase levels are increased in childhood, and are typically considerably more elevated than in GSD I. Also, in contrast to GSD I, blood lactate and uric acid concentrations are normal. Untreated infants and children grow slowly and puberty is delayed. The kidneys are not enlarged and renal dysfunction does not occur. In type IIIa, muscle weakness is usually minimal and not clinically significant in childhood. Myopathy usually becomes prominent in the third or fourth decades of life manifesting as slowly progressive muscle weakness involving the large proximal muscles of the shoulders and hips [68]. Patients may also have involvement of the distal muscles; e.g., the small muscles of the hand and, in some cases, this is associated with peripheral neuropathy [69]. Limit dextrin may also accumulate in the heart causing a cardiomyopathy that is echocardiographically similar to idiopathic hypertrophic cardiomyopathy [70,71]. Hepatic adenomata occur in 25% of patients [72]. With the exception of myopathy, symptoms and signs characteristically ameliorate with increasing age. The size of the liver tends to decrease to normal during puberty; however, most patients show hepatic fibrosis on biopsy and, rarely, adult patients develop cirrhosis and its complications [73].

Diagnosis

The principal biochemical abnormalities are shown in Table 2. Ketotic hypoglycemia without hyperlacticacidemia occurs with fasting. Glucagon does not elicit a glycemic response when given after a fast, but does when given 2 hours after a carbohydrate-rich meal. Elevated levels of serum creatine kinase and aldolase concentrations suggest muscle involvement, but normal values do not exclude myopathy. Electromyography shows myopathic changes and ischemic forearm muscle testing reveals a smaller than expected increase in blood lactate concentration. Liver histology reveals glycogen storage; fibrosis may be prominent, but fat infiltration is not typical. Muscle histology shows free glycogen, which is periodic acid Schiff (PAS) positive and digestible by diastase, and on electron microscopy appears as normal particles. A definitive diagnosis is obtained by demonstrating abnormal glycogen (limit dextrin with short outer branches) in liver and/or muscle and deficiency of debranching enzyme activity. Definitive subtyping of GSD III formerly required biopsies of both liver and muscle; however, the striking and specific association of exon 3 mutations with type IIIb now allows subtyping of GSD III using DNA obtained from blood [62]. Mutation analysis is not yet available for the diagnosis of GSD IIIa.

Management

As in GSD I continuous provision of an adequate amount of glucose using uncooked cornstarch, 1.75 grams per kg at six hour intervals during both day and night, maintains normoglycemia, increases growth velocity, and decreases serum transaminase concentrations [74,75]. Continuous nocturnal feeding of a nutrient mixture consisting of glucose or glucose oligosaccharides, and protein or amino acids, combined with intermittent high protein feedings during the day may be especially beneficial for patients who have significant growth retardation and myopathy [76,77]. Protein can be used as a substrate for gluconeogenesis, which is intact in GSD III [78]. Milk products and fruit should not be restricted as galactose and fructose can be normally converted to glucose.

As in GSD I, annual serum α-fetoprotein determinations and hepatic ultrasound examinations are obtained to screen for hepatic adenomas. Malignant transformation of hepatocellular adenomas is rare, but has been reported in GSD IIIa. Liver transplantation has been performed in patients with end-stage cirrhosis and/or carcinoma [79,80]. In the small number of patients who have had a liver transplant, metabolic parameters improved but muscle disease was not beneficially affected [79]. Patients with muscle disease should have intermittent cardiac evaluations, including EKGs and echocardiograms. The prognosis is favorable for the purely hepatic form (IIIb), but is less favorable for GSD IIIa, as severe myopathy and cardiomyopathy may develop even after a long period of apparent latency. Currently, there is no satisfactory treatment for the progressive myopathy. Exercise causes elevation in serum creatine kinase and aldolase concentrations and it has been suggested that restricting exercise may slow progression of muscle damage.

GLYCOGEN BRANCHING ENZYME DEFICIENCY (TYPE IV GSD; ANDERSEN DISEASE; AMYLOPECTINOSIS)

GSD IV is caused by deficient glycogen branching enzyme (GBE, amylo-1,4 to 1,6-transglucosidase) activity. This enzyme catalyzes the transfer of α-1,4-linked glucosyl units from the outer end of a glycogen chain to an α-1,6 position on the same or a neighboring glycogen chain. Branching is essential to pack a large number of glucosyl units into a relatively soluble spherical molecule. GBE deficiency causes accumulation in the liver of an abnormal glycogen molecule with few branch points and long α-1,4-linked glucose polymers resembling amylopectin. The abnormal glycogen acts as a foreign body and induces cirrhosis.

GSD IV accounts for about 3% of all cases of GSD. It is inherited as an autosomal recessive trait. Mutations in the same glycogen-branching enzyme gene, located on chromosome 3p12, are responsible for both the hepatic and neuromuscular forms of the disease. A genotype-phenotype correlation has been established for the more common mutations and may help to predict prognosis in individual cases. Absent enzyme activity is associated with a severe disease; milder phenotypes have residual enzyme activty [81].

Clinical Features

GSD IV typically presents in early infancy with hepatosplenomegaly and failure to thrive. As non-branched glycogen is available for glycogenolysis, hypoglycemia is unusual in GSD IV until late in the disease when cirrhosis is advanced. The typical clinical course is rapidly progressive liver cirrhosis with portal hypertension, esophageal varices and ascites, culminating in death from liver failure usually by five years of age [82]. Hepatocellular carcinoma may develop [83]. Accumulation of amylopectin-like polysaccharide in cardiac muscle can result in a fatal cardiomyopathy.

The less common neuromuscular form of GSD IV is clinically and genetically heterogeneous. Four main phenotypic variants have been described based on the age of onset [84]. 1. A perinatal form with fetal akinesia deformation sequence characterized by multiple congenital contractures, hydrops fetalis, and perinatal death [85]. 2. A congenital form with congenital hypotonia, muscle atrophy, and weakness, and rapid deterioration with death in early infancy [86,87]. 3. A late childhood-onset variant that presents with skeletal myopathy or cardiomyopathy [88,89]. 4. A milder adult-onset form that presents as an isolated myopathy or with central and peripheral nervous system involvement resulting from accumulation of unbranched glycogen in neuronal tissue (adult polyglucosan body disease) [90]. These patients have upper and lower motor neuron involvement and progressive dementia [91].

Diagnosis

The diagnosis is established by demonstrating abnormal glycogen (with long outer chains, an amylopectin-like abnormal polysaccharide) that stains with PAS but is partially resistant to diastase digestion. Electron microscopy shows fibrillar aggregations of glycogen in addition to normal appearing glycogen arranged in a and b particles. Hepatic fibrosis and cirrhosis are seen in the classic form of the disease. In the neuromuscular forms, serum creatine kinase is elevated. Branching enzyme is deficient in liver, muscle, leukocytes, erythrocytes, or fibroblasts. The diagnosis is confirmed by demonstrating absent branching enzyme activity in skin fibroblasts. In adult polyglucosan body disease, the branching enzyme deficiency can only be detected in leukocytes or in a nerve biopsy.

Treatment

There is no specific treatment for GSD IV. The onset of cirrhosis can be rapid; affected infants should be promptly referred to a liver transplant center. For progressive liver failure, transplantation has been an effective treatment and has resulted in reduced glycogen storage in both heart and skeletal muscle [79,92].

GLYCOGEN PHOSPHORYLASE DEFICIENCY (TYPE VI GSD; HERS DISEASE) AND PHOSPHORYLASE KINASE (PHK) DEFICIENCY (TYPE IX GSD)

Glycogenoses caused by a reduction in liver phosphorylase activity are a heterogeneous group of disorders (Table 1) of which deficiency of phosphorylase *b* kinase (PHK), resulting in failure of hepatic phosphorylase activation (Figure 2), is the most common, accounting for about 25% of all cases of GSD [93]. Deficiency of hepatic phosphorylase itself (PYGL) is rare [94] except in the Mennonite community in which 0.1% of individuals have the disease [94,95].

PHK stimulates glycogenolysis by phosphorylating and thereby activating glycogen phosphorylase (Figure 2). PHK of liver and muscle is a complex enzyme consisting of four subunits: α, β, γ, and δ, each encoded by a distinct gene. The holoenzyme consists of 4 copies of each isoform, for a final complex of 16 subunits. The disorder is genetically heterogeneous, with both autosomal recessive and X-linked forms (Table 1), which explains why there are different classifications. Mutations in three different genes of PHK subunits (PHKA2, PHKB and PHKG2) can result in deficient hepatic phosphorylase activity (Table 1).

X-linked glycogenosis (XLG), caused by mutations in the gene encoding the liver isoform of the PHK α subunit (PHKA2), is the most common variant (about 75% of all cases). Numerous different mutations in PHKA2 have been identified in XLG [96-98]. The enzyme is lacking in liver but is normal in muscle. In XLG subtype II, PHK activity is low in liver but is normal or increased in erythrocytes and leukocytes [98-100]. Autosomal liver disease is caused by a mutation in the catalytic g subunit encoded by PHKG2 gene at 16 p12 [101]. These patients are at risk of a more severe fibrotic liver disease. Muscle-specific disease is caused by mutations in the muscle-specific α subunit (PHKA1) located at Xq13 [102].

Patients with glycogen phosphorylase deficiency are clinically indistinguishable from those with liver phosphorylase *b* kinase deficiency. Furthermore, mutations in PHKA2, PHKB, and PHKG2 all cause a similar clinical phenotype.

Clinical Features

These disorders are milder than GSD I and III and generally have a good prognosis. Presentation is usually in infancy or early childhood with growth retardation, hepatomegaly, and a protuberant abdomen. Symptomatic hypoglycemia and ketosis is unusual except with prolonged fasting or strenuous physical exercise. Blood lactic acid and uric acid concentrations are normal and metabolic acidosis is rare. Mild hypertriglyceridemia, hypercholesterolemia, and elevated serum transaminase levels may be present. Motor development may be delayed as a consequence of muscular hypotonia in the autosomal recessive form of the disorder with reduced enzyme activity in both muscle and liver. The clinical course is usually benign. Clinical and biochemical abnormalities gradually disappear

with increasing age. Hepatomegaly decreases at puberty and most adult patients are asymptomatic [103]. Patients have a growth pattern characterized by initial growth retardation between 2-10 years of age, a delayed pubertal growth spurt, with complete catch-up in final height [104]. Uncommon clinical phenotypes have been described, including renal dysfunction with proximal renal tubular acidosis [105], central nervous system abnormalities (seizures, delayed cognitive and speech abilities, peripheral sensory neuropathy) [98], and progression to cirrhosis in childhood in the liver-specific subtype [101,106,107]. Fatal infantile cardiomyopathy has been described in children [108]. Myopathy presents with exercise intolerance, cramps, myalgias, muscle weakness, myoglobinuria and, in rare cases, hypotonia in young children. In the adult-onset form (autosomal inheritance), progressive distal muscle weakness is more prominent than proximal muscle weakness [109-111].

Diagnosis

Table 2 shows the principal biochemical abnormalities. Unlike GSD I and III, the response to glucagon is usually normal. Diagnosis of glycogen phosphorylase deficiency is possible by assaying phosphorylase activity in purified blood cell fractions. Phosphorylase kinase *b* also can be measured in leukocytes and erythrocytes. In liver PHK deficiency, activity of the enzyme is usually low in erythrocytes, thus allowing a biochemical diagnosis to be made from a blood sample. Normal phosphorylase *b* kinase activity in erythrocytes does not definitively rule out type IX GSD because PHK activity is deficient in liver, but normal or even increased in erythrocytes in the less common variant of liver PHK deficiency designated X-linked liver glycogenosis subtype II. Because phosphorylase activity is influenced by multiple allosteric effectors, as well as by humoral and neural signals that are difficult to control, it may be difficult to determine by enzymatic analysis whether a defect in the liver phosphorylase system is due to a deficiency of phosphorylase itself or deficiency of phosphorylase kinase. Furthermore, phosphorylase *b* kinase deficiency is accompanied by decreased total phosphorylase activity. For these reasons, molecular diagnosis by direct sequencing should be performed whenever possible [1].

Management

Prolonged fasting should be avoided. A bedtime snack may be sufficient to prevent morning hypoglycemia, but ketosis is prevented and patients often feel better with uncooked cornstarch supplementation prior to bedtime (1.5 – 2 grams/kg) [112]. Improved growth has also been reported in children receiving cornstarch supplementation.

GLUCOSE TRANSPORTER-2 DEFICIENCY (FANCONI-BICKEL SYNDROME; GLUT2 DEFICIENCY; GSD XI)

Fanconi-Bickel syndrome (FBS) is a rare autosomal recessive disorder due to mutations in the GLUT2 gene located at 3q16.1-q26.3 [113]. A total of 33 mutations have been described. GLUT2 is a facilitative monosaccharide transporter that mediates transport of D-glucose and, to a lesser extent, D-galactose across the cell membrane of hepatocytes, pancreatic ß cells, and the basolateral membrane of renal proximal tubular cells and enterocytes [7]. GLUT2 is different from other members of the facilitative glucose transporter family: it is insulin-independent and has a high K_m (~ 40mmol/L), which means that glucose transport by pancreatic ß-cells and hepatocytes is proportional to the blood glucose concentration. This permits these cells to sense the prevailing glucose concentration via the activity of glucokinase, which in turn leads to control of insulin secretion by the pancreas and uptake or release of glucose by hepatocytes as required to regulate the blood glucose concentration [7].

Clinical Features

The clinical syndrome was designated GSD XI, but use of this designation is no longer favored since the originally proposed functional defect has proven to be incorrect. FBS is a glycogen storage disease that shares several clinical features with both GSD 0 and GSD I. It was first described in a 3-year-old Swiss boy in 1949 [114] and since then more than 110 cases have been reported from Europe, Israel, Japan, Northern Africa, the Middle East, and North America [115]. Deficiency of GLUT2 is characterized by glucose and galactose intolerance and accumulation of glycogen in the liver and kidney. As with the other GSDs, presentation typically is in infancy when the intervals between overnight feeds increase [114]. Nocturnal irritability and morning lethargy are characteristic features. Patients may present with chronic diarrhea (from carbohydrate malabsorption), failure to thrive, and developmental delay. Presence of a "moon facies" and a protuberant abdomen may lead to confusion with GSD I. Short stature is almost universal in FBS and persists into adulthood [115,116].

Abnormal hepatocyte glucose transport and diminished glucose-stimulated insulin release results in postprandial hyperglycemia, which can easily be confused as early diabetes mellitus. Fasting hypoglycemia is due to abnormal glucose transport out of the liver, impaired glycogenolysis secondary to increased intracellular glucose concentrations, and renal glucose wasting from impaired renal proximal tubular glucose reabsorption. Some clinical features overlap with GSD Ia (hepatomegaly, nephromegaly, hypoglycemia); however, patients with GSD Ia have pronounced fasting lactic acidosis with much less pronounced ketonemia. Persistent glucosuria is another distinctive clinical feature that differentiates FBS from GSD Ia. Fasting hypoglycemia and postprandial hyperglycemia may also be confused with GSD 0. The absence of hepatomegaly in the latter disorder, however, is a major distinguishing feature.

In FBS there is a characteristic tubular nephropathy with glucosuria, phosphaturia, bicarbonate wasting, and a generalized aminoaciduria, leading to rickets [117]. Osmotic diuresis causes polyuria. The disorder also has been detected by finding increased blood levels of galactose on newborn screening for galactosemia [118,119].

Short stature and osteopenia are common in untreated children, but improve by preventing hypoglycemia, acidosis, and ketosis. Despite recurrent hypoglycemia, neurologic impairments and seizures are uncommon, probably due to the availability of alternative metabolic substrates with fasting. Hepatic adenomas have not been reported in this disorder, but a renal disease with focal segmental glomerulosclerosis and microalbuminuria, similar to that seen in GSD I, has been reported [115,120].

Diagnosis

The diagnosis of FBS should be considered when postprandial hyperglycemia alternates with fasting ketotic hypoglycemia. Recommended screening tests include a glucose or galactose tolerance test and studies of kidney function looking for glucosuria and evidence of proximal tubular dysfunction. Mutation analysis can be used to confirm the diagnosis [121]. Liver biopsy reveals increased glycogen content without significant inflammation or fibrosis, but is no longer required for diagnosis.

Management

The goal of treatment is to prevent hypoglycemia, normalize plasma glucose concentrations, and minimize systemic acidosis. No specific treatment is available. Frequent small meals during the day supplemented with uncooked cornstarch, 1.5-2 gram per kg b.i.d., improves growth and stamina [122]. Because fructose transport into cells is facilitated by GLUT5, fructose can be used as an alternative source of carbohydrate. High concentrations of glucose, sucrose, and galactose are avoided because they exacerbate hyperglycemia and aggravate malabsorption. Management of the renal disease consists of supplementation of water, electrolytes, bicarbonate, and vitamin D. Acute decompensation can occur during surgery, and careful monitoring is required whenever patients are required to fast or when counterregulatory mechanisms are activated by stress.

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Chapter XI

LIVER TRANSPLANTATION FOR METABOLIC DISEASE

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ABSTRACT

Liver transplantation (LT) is commonly used to treat acute and chronic liver failure in the United States. Currently, more than 4,000 LTs are performed yearly in the United States [1]. LT is effective for a number of metabolic liver diseases. The most common pediatric metabolic liver disorders treated with LT in children are α_1 – Antitrypsin deficiency, Wilson disease, neonatal hemochromatosis, hereditary tyrosinemia, and glycogen storage disorders. Among adults, α_1 – Antitrypsin deficiency, Wilson disease, hemochromatosis and increasingly, nonalcoholic fatty liver disease are the most common metabolic diseases treated with LT although much less common than among pediatric groups. In the USA, metabolic diseases account for less than 4% of adult LT and approximately 20% of pediatric LT. The results of LT for metabolic diseases are generally excellent with some exceptions, notably among patients with hemochromatosis, as described below. Overall, adults have a 1 year survival rate of 88% and 3 year survival rate 84% after LT for metabolic disease [1,3]. One-year survival of 94% and 5 year survival 92% has been reported among children [2,3]. Among 40,000 LT in a 13 year period recorded in the European transplant registry, 6% were performed for metabolic diseases [26]. Cumulative patient survival rates were 79% at 1 year and 70% at 5 years. It is possible that graft and patient survival rates have improved further in recent years. In a single center study of LT or combined LT/kidney transplantation (KT) for metabolic diseases, an excellent 1 year survival rate of 92% was reported after LT and 91.8% after combined LT/KT [27]. Therefore, LT is a successful and frequently definitive therapy for many metabolic diseases associated with the liver.

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Metabolic disorders treatable by LT may be classified into four categories: (Table 1).

Table 1. Metabolic disorders treatable by liver transplantation.

I. *Primary metabolic defect is in the liver*
A. *Liver transplantation primarily for hepatic complications*
Wilson disease
α_1 – Antitrypsin deficiency
Hereditary tyrosinemia type 1
Glycogen storage disease type I and IV
Galactosemia
Progressive familial intra-hepatic cholestasis
Alagille's syndrome
Neonatal hemochromatosis
B. *Liver transplantation primarily for extra-hepatic complications*
Primary hyperoxaluria type I (with kidney co-transplantation)
Familial hypercholesterolemia (with cardiac transplantation)
Crigler-Najjar syndrome type I
Urea cycle defects
Hemophilia A and B
Hereditary Protein C deficiency
Familial Amyloidotic Polyneuropathy
Hereditary protein C deficiency
II. *Primary defect is extra-hepatic*
A. *Liver disease may recur after transplantation*
Hereditary Hemochromatosis
Gaucher disease
Familial Erythropoietic Protoporphyria
Nonalcoholic Steatohepatitis
Cryptogenic cirrhosis
B. *Liver transplantation curative for hepatic component of generalized disorder*
Cystic fibrosis

Adapted with permission from Tung, BY, Kowdley, KV: Liver transplantation for Hemochromatosis, Wilson disease, and other metabolic disorders. Clinics in liver disease Vol 1, no 2 August 1997 341-360 [34].

I A. Disorders in which the liver is the primary site of metabolic dysfunction and LT is undertaken primarily for treatment of hepatic complications. LT in this situation not only replaces a dysfunctional liver but also corrects the underlying metabolic disorder.

I B. Disorders in which LT is undertaken primarily to correct the underlying metabolic disorder which causes severe extra-hepatic organ dysfunction while liver function is preserved. Simultaneous transplantation of other affected organs may also be necessary.

II A. Disorders in which the liver is not the site of the primary metabolic defect. LT replaces the affected liver but in these disorders, transplantation may not be curative and disease may recur in the transplanted liver.

II B. Disorders in which liver disease is part of a generalized metabolic defect. LT is curative for the hepatic component of the defect but has minimal effect on the extra-hepatic manifestations of the disease.

I A. Primary Metabolic Defect in Liver: Liver Transplantation Primarily for Hepatic Complications

Wilson Disease

Wilson disease (WD) is an autosomal recessive disorder of copper metabolism that leads to reduced biliary excretion of copper and progressive accumulation of copper in various tissues. The estimated prevalence is 1:30,000 to 1:55,000 depending on the method of analysis [4,5] with a gene frequency of 1:90 to 1:150. The WD gene *ATP7B* localized [24] to chromosome 13, encodes an intracellular copper transporting P-type ATPase that localizes predominantly to the trans-Golgi apparatus of hepatocytes. Over 200 mutations of *ATP7B* are reported and many patients with WD have two different mutations of the gene on each allele encoding the WD gene (compound heterozygotes). Defective ATP7B protein function results in reduced vesicular secretion of copper into bile and also reduced incorporation of copper into synthesized apoceruloplasmin [6-8]. The resulting copper accumulation in the liver leads to progressive liver dysfunction, cirrhosis and may present as fulminant liver failure. Excess hepatic copper is released into circulation as free serum copper which may accumulate in the brain, eyes, kidneys, heart, ovaries and musculoskeletal system. The clinical manifestations of WD are protean and include symptoms and signs of acute or chronic liver disease, as well as neuro-psychiatric symptoms, Kayser-Fleischer (KF) rings, kidney disease with hypercalciuria, aminoaciduria and nephrocalcinosis, cardiac disease, hemolytic anemia, infertility, amenorrhea, hypoparathyroidism, osteoporosis, osteoarthritis and chondrocalcinosis. The age at the time of presentation ranges from 5 years to over 80 years of age [9]; about 50% of patients present by 15 years of age. The diagnosis of WD is established by a combination of clinical, biochemical and pathological criteria. WD should be considered in all patients with unexplained liver disease especially in the presence of neuro-psychiatric symptoms. The presence of KF rings in the presence of a low serum ceruloplasmin can confirm the diagnosis of WD [25]. In the absence of KF rings, a low serum ceruloplasmin and hepatic copper content greater than 250µg/g dry weight suggest the diagnosis of WD. Serum free copper greater than 25µg/dl, a 24 hour urinary copper over 100µg (normal is 20–50 µg, in untreated WD it ranges from 100 to 1000 µg) and genetic analysis for ATP7B mutations may also aid in diagnosis. As an adjunct, urinary copper excretion after two 500 mg doses of D-Penicillamine 12 hours apart may provoke brisk copper excretion at >1600µg/24 hours in patients with WD.

Liver Disease

Some degree of liver disease is noted in most patients with WD. The changes may vary from nonspecific changes to micro-vesicular and macro-vesicular steatosis, chronic active hepatitis, fibrosis and cirrhosis. Hepatocellular malignancies although uncommon, are reported in patients with WD [29]. Liver disease generally precedes neuro-psychiatric manifestations by many years. Hepatic disease may manifest as fulminant hepatic failure with hemolytic anemia, anorexia, malaise, nausea, abdominal and right upper quadrant pain, jaundice, spider angiomas, anasarca, ascites, bacterial peritonitis, esophageal varices, splenomegaly, malnutrition, delayed puberty, gynecomastia and amenorrhea.

Chronic hepatitis is the most common presentation of hepatic WD and is indistinguishable from chronic hepatitis from other causes [10]. Clinical symptoms are nonspecific; jaundice and KF rings may be absent and serum ceruloplasmin may be normal or even elevated as an acute phase reactant. It is necessary to quantitate hepatic copper content since liver biopsy specimens may not show stainable copper. Progressive liver failure may follow rapidly without treatment but life expectancy may be normal with early diagnosis and initiation of copper chelation therapy [11,15].

Acute hepatitis and fulminant liver failure, although infrequent in adults, is the most common presentation of hepatic WD in children and adolescents [12,13,14] and is more common in females (Female: Male ratio, 5:1). Acute hepatitis may be self limited but may progress to fulminant hepatic failure. In the setting of acute or end-stage liver failure, the diagnosis of WD may be difficult to establish. Although copper quantitation in liver biopsy is the gold standard test, liver biopsy is usually contraindicated because of coagulopathy. Ceruloplasmin may be low in any cause of fulminant liver failure or may be increased into the normal range as an acute phase reactant. In patients presenting with fulminant hepatic failure, the combination of Coombs'- negative hemolytic anemia, elevated bilirubin, modest elevations of aminotransferases and normal to mildly elevated alkaline phosphatase levels should raise clinical suspicion of acute WD [13,14]. Fulminant hepatic WD may be fatal without transplantation [13,14,16,17].

Neuro-Psychiatric Disease

Neuro-psychiatric symptoms and signs of WD typically follow liver disease by more than 5 years and usually after the second decade of life. Liver disease may be asymptomatic in such patients. Neurological symptoms include tremor and other involuntary movements, lack of muscle co-ordination, micrographia, drooling, dysarthria, muscle rigidity, pseudobulbar palsy, dysphagia and headaches. Associated psychiatric symptoms may include insomnia, anxiety, depression and personality changes. Behavioral changes, especially in children, may accompany worsening performance in academic or athletic activities [23].

Medical Therapy

The medical treatment of WD has been reviewed in detail in several recent publications [22,28]. Generally, treatment consists of therapy with Zinc or Trientine or D-Penicillamine or a combination of either Trientine or D-Penicillamine with Zinc [15,22]. Trientine has increasingly replaced D-Penicillamine as the first line chelating agent. Lifelong therapy is necessary to mobilize excess hepatic and systemic copper and to prevent its re-accumulation.

Zinc is used in patients diagnosed early without significant end organ damage or for maintenance therapy (in patients with end organ damage) after negative copper balance has been achieved with chelation therapy. Recent studies have examined Ammonium tetrathiomolybdate [not FDA approved] in neurological WD given that neurological deterioration is least with Tetrathiomolybdate (5%) in comparison to Trientine (20%) and Penicillamine (50%) [22,28].

Liver Transplantation

Indications for LT in WD include fulminant hepatic failure, liver dysfunction unresponsive to chelation therapy, advanced liver disease after non compliance with chelation therapy despite history of previous response to chelation therapy and presence of cirrhosis [16,19]. Although LT is not indicated as primary treatment of Wilsonian neurological disease, neuro-psychiatric manifestations of WD may improve after LT for decompensated or acute hepatic WD [18,19,20,21]. While awaiting LT, especially in fulminant hepatic failure, plasmapheresis or albumin dialysis may lower circulating copper released by massive hepatocellular lysis.

Survival after LT for WD is acceptable. Bellary et al [16] reported single center results after LT on 39 patients, 22 with fulminant hepatic failure and 17 with chronic liver disease. Overall 1 year survival was 79%, 90% for those with chronic liver disease and 73% for those with a fulminant hepatic failure. Geissler et al [18] report six patients (three females and three males) who underwent LT for WD. During follow-up ranging from 3 to 7 years, all patients were alive with functioning allografts. Serum ceruloplasmin levels increased after transplantation and remained normal. Neuro-psychiatric manifestations improved significantly in two of these patients. Emre et al [31] report their experience between 1988 and 2000 with 21 LTs performed in 17 patients with WD, at a mean age of 28 years (range 4-51 years). Eleven patients had fulminant hepatic failure and six had chronic liver disease. Renal failure, present in 45% of patients with fulminant WD, resolved post-LT with supportive care. One-year patient and graft survival was 88% and 63%, respectively. Sutcliffe et al [32] prospectively followed 24 patients who underwent LT for WD. Indications for LT included acute liver failure in 15 patients, sub-acute liver failure in three, and chronic liver disease in six. There were three deaths, all between 1988 -1993, one of whom had multi-organ failure before LT and died within 24 hr of surgery and two patients died within 1 year due to immunosuppressant-related complications. After a median follow-up of 92 months, all survivors had satisfactory graft function (5-year patient and graft survival, 87.5%), with quality-of-life scores in a majority (86%) of survivors comparable to matched controls from the general population.

Living donor LT (LDLT) has also been performed for WD. Tamura et al [33] recently reported 5 living related liver transplants including 2 patients with fulminant hepatic failure and 3 with chronic liver disease. One patient died from early graft thrombosis and the surviving 4 patients had excellent clinical and biochemical improvement over the 2 year follow-up period. Wang et al [21] report a series of 22 patients between 2001 and 2003 who received LDLT. A total of 19 pediatric patients and 3 adults of whom 20 had chronic liver disease and 2 had co-existent fulminant hepatic failure received LDLT. Neurological manifestations were present in 9 of the 20 with chronic liver disease. Long term survivors

(21/22) reportedly enjoyed normal health, good quality of life, significant improvement in neurological symptoms after a mean follow-up period of 18.5 months (range 4–38 months).

LT is life saving and in the long term, reverses most of the metabolic abnormalities associated with WD [16,21,31-33,36]. Serum ceruloplasmin and copper measurements normalize post-transplant and long-term copper chelation therapy is not needed. Significant hepatic copper re-accumulation has not been described in patients transplanted for WD. Kayser-Fleischer rings disappear in most, but not all, patients receiving LT. In most of the series described above, surviving patients with preoperative neurological symptoms had some degree of neurological improvement after transplantation. LT has rarely been performed for severe neurological WD in the absence of significant hepatic dysfunction [18-21]. However this remains a controversial indication for LT.

Alpha-1-Antitrypsin Deficiency

Alpha-1-antitrypsin (AAT) deficiency is an autosomal recessive disorder first described in the 1960s by Laurell and Eriksson in patients with severe pulmonary emphysema [62]. It affects 1 in 1,550 live births in Northern Europe to 1 in 2800 in North America, New Zealand and Australia [65]. Worldwide estimates of roughly 116 million carriers and 1.1 million subjects with severe AAT deficiency suggest that AAT deficiency is a prevalent but under-recognized hereditary disorder [65].

AAT is a 52 kD glycoprotein secreted into blood by hepatocytes, pulmonary epithelial cells and phagocytes. It irreversibly inhibits a variety of serine proteases, including cathepsin G, and proteinase, and predominantly targets human neutrophil elastase [72]. With severe deficiency or absence of AAT, increased destruction of the pulmonary connective tissue matrix results in premature emphysema. In contrast, hepatic disease arises not from the deficiency of the protease inhibitor but from progressive accumulation of abnormally polymerized and folded AAT in the endoplasmic reticulum of hepatocytes. Low plasma concentrations of AAT result from this lack of secretion of AAT from hepatocytes. These aggregates of abnormal AAT are easily visualized by Periodic Acid–Schiff (PAS) staining and electron microscopy [66,72]. The nomenclature to identify AAT variants evolved from different techniques applied to study the protein over the last 40 years. AAT variants were included in an allelic Pi (protease inhibitor) system and were initially named based on their migration velocity in starch-gel electrophoresis as F (fast), M (medium), S (slow) or Z (very slow) [69]. The former Pi system was renamed PI* and subsequently, AAT variants were classified into three major clinically relevant categories [70,71].

Normal: This category includes the four most common M variants (M1 to M4) and a number of less common variants. AAT plasma levels are normal (85-215mg/dl) and there is no risk of lung or liver disease.

Deficient: This category includes the most common Z and S variants and a number of less frequent variants including M-like variants with a middle migrating pattern. AAT plasma levels, are reduced (maximum AAT level in this group is 80mg/dl), significantly increasing the risk of lung or liver disease.

Null or QO: There is no detectable plasma AAT level, associated with an increased risk of developing emphysema but not liver disease.

Of the numerous mutations that could result in a partial deficiency of AAT, the S and the Z mutations are most prevalent. Homozygosity for the common S mutation (Glu264Val) results in a 40 percent decrease in plasma AAT levels. However, homozygosity for the Z mutation (Glu342Lys) results in a severe (85%) deficiency of plasma AAT. ZZ homozygotes and SZ compound heterozygotes may develop severe emphysema while SS homozygotes do not develop significant disease [72].

In the neonatal and pediatric population, AAT deficiency is now recognized as the most common cause of inherited liver disease and the most common genetic indication for LT [12]. Approximately 10% of those with AAT deficiency develop significant liver disease in the form of chronic active hepatitis, cryptogenic cirrhosis and portal hypertension, by their fourth decade of life [73]. There is also an increased risk of developing hepatocellular carcinoma (HCC) particularly among men [74].

In adults, AAT deficiency must be suspected in any patient who presents with unexplained chronic liver disease or HCC. In neonates, liver disease first presents during 4 to 8 weeks of age as persistent cholestatic jaundice. Most improve spontaneously and are asymptomatic by 1 year of age. Among symptomatic patients, jaundice, elevated serum aminotransferases, hepatomegaly, pruritus, hypercholesterolemia, severe liver dysfunction, chronic active hepatitis, cryptogenic cirrhosis, portal hypertension, splenomegaly and HCC may be observed. In addition, neonates may present with bleeding diathesis in the form of hematemesis, melena, bleeding from the umbilical stump, or bruising; however, AAT rarely manifests severe liver injury during infancy [73]. The diagnosis is confirmed by demonstrating low serum AAT levels (lower limit of normal 85 mg/dL), confirmation of an abnormal AAT phenotype [protease inhibitor type (PI type)] and evidence of eosinophilic, PAS positive, diastase-resistant globules in liver biopsy specimens. It is important to note that AAT level alone is insufficient to exclude or make the diagnosis because serum AAT may be elevated as an acute phase reactant or may be low because of decreased hepatic synthesis.

Treatment of lung disease in AAT deficiency is supportive. Cigarette smoking accelerates emphysema and must be avoided. In those with progressive emphysema, replacement therapy with intravenous or aerosolized purified plasma or recombinant AT may be considered [75]. Severe emphysema from AT deficiency can be treated with lung transplantation [76].

There is no proven medical therapy for AAT deficiency-associated liver disease. Treatment is focused on management of complications of chronic liver disease and LT should be offered to patients with end stage liver disease. AAT deficiency is the most common inherited liver disease for which LT is performed in children. Between 1990-1999, 76 US centers reported 551 liver transplants for metabolic liver disease of which AAT deficiency was the most common indication (n=261) [2].

Roughly 10% of children who initially present with neonatal cholestasis eventually require LT [73,80] with the mean age at LT ranging from 4.6 to 10.6 years [80-84]. Although early reports of 57% 1-year survival post LT for AAT deficiency were disappointing, more recent reports reflect excellent prognosis with 94% 1-year and 92% 5-year survival [81-85].

LT has also been performed for adults with AAT deficiency. In a series of 22 adults transplanted for AAT deficiency, the following AAT phenotype patterns were observed: three were PIZZ; nine-PIMZ; three-PIMM; two PIMS; and one-PISZ; AAT phenotype was not reported in 4 patients. Although liver biopsy revealed periodic acid-Schiff-positive, diastase-resistant globules suggestive of AAT accumulation in all patients, 10 patients also had significant alcohol history and two had evidence of chronic viral hepatitis. Overall post LT 1-year survival was 68% and improved to 73% for those transplanted after 1990 [80]. In an earlier series of eight PIZZ and two PIMZ adults, post LT 1-year survival was 60% [81].

LT cures AAT deficiency; the AAT phenotype changes to that of the donor after LT and serum levels of AAT improve to the normal range [82,89]. In one case where a recipient acquired a PIZZ phenotype via a liver transplant from an asymptomatic PIZZ donor, the recipient remained asymptomatic over a 6 year follow-up period although a delayed rise of liver enzymes in a cholestatic pattern, chronic portal hepatitis and fibrosis associated with AAT deposits were noted [90]. The effects of LT on pulmonary function have not been studied in detail. Over a 1-6 year follow-up, post-LT forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) ratio greater than 70% was noted in 8 of 10 patients, but comparative pre-transplant pulmonary function testing and smoking history were not reported [80]. Hepatocyte transplantation has been studied in mouse models and AAT deficiency may be a good candidate for further studies of gene replacement therapy in the future [73].

Hereditary Tyrosinemia Type I

Tyrosinemia type I (TT1) is an autosomal recessive disorder and the most common disease caused by defects in tyrosine metabolism. A mutation in the gene for fumaryl acetoacetate hydrolase (FAH), the terminal enzyme catalyzing tyrosine degradation, causes FAH deficiency and results in accumulation of the intermediate metabolites maleyl- and fumaryl- acetoacetate which are hepatotoxic. Secondary metabolites such as succinylacetoacetate and succinylacetone may have both local and systemic adverse effects including the inhibition of porphobilinogen synthase and porphyria- like neurologic crises [91,92].

The clinical presentation is variable, even within the same family [91-93]. Acute liver failure with jaundice, ascites, coagulopathy, encephalopathy and hypoglycemia due to liver failure or hyperinsulinemia, is a common presentation in infants within the first 6 months of life. Older infants may have failure to thrive, hypotonia, rickets, coagulopathy and hepatosplenomegaly. After infancy, chronic liver disease, cardiomyopathy, renal failure or a porphyria-like neurologic crisis with self mutilation may occur. Renal tubular dysfunction and hypophosphatemic rickets may manifest at any age. Liver disease leads to cirrhosis and hepatocellular dysplasia with a high incidence of HCC.

Serum aminotransferases, bilirubin, and alpha fetoprotein are elevated and plasma tyrosine, phenylalanine and methionine are usually more than 3 times normal. Urinary succinyl acetone may be elevated and renal tubular dysfunction may cause aminoaciduria and phosphaturia. Radiographs may reveal hypophosphatemic rickets and echocardiography may show hypertrophic cardiomyopathy. Liver biopsy findings are nonspecific and may

demonstrate steatosis, increased iron and cirrhosis. Hepatocyte dysplasia is common and HCC may frequently be present on radiologic imaging or in explant livers [97-99].

Prognosis and survival improve with older age at onset of symptoms; infants presenting within the first 2 months of life have only a 30% 1-year survival, while 1-year survival is 75% for those presenting from 2 to 6 months, and greater than 90% for those presenting after 6 months of age [93]. Dietary restriction of phenylalanine and tyrosine along with supportive measures can ameliorate the symptoms and some improvement of hepatic and renal function can be expected. Oral administration of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), an inhibitor of 4-Hydroxy phenylpyruvate dioxygenase in the tyrosine catabolic pathway prevents formation of maleylacetoacetate and fumarylacetoacetate and their conversion to more toxic metabolites. NTBC therapy has greatly improved outcomes in TT1 with some children showing normal growth up to 12 years. There may be a decreased need for or delay of LT in TT1 patients treated with NTBC and diet but long-term results of NTBC therapy, especially regarding risk of HCC, are yet to be reported [94,99].

Indications of LT in TT1 include acute liver failure, decompensated chronic liver disease, evidence of hepatic dysplasia or HCC or impaired quality of life. LT for TT1 has been successfully undertaken in several centers, but may be complicated by the presence of HCC [95-97,99,100]. The prevalence of HCC has been reported to be as high as 25% to 50% in TT1 liver explants [97,98]. Most urinary and serum markers of abnormal tyrosine metabolism return to normal after transplantation, but proximal renal tubular dysfunction may persist due to ongoing renal expression of abnormal FAH [95,96,99,100]. In one report of 3 patients, hypertrophic cardiomyopathy resolved and refractory hypoglycemia resolved in one patient [100]. Phenylalanine and tyrosine restriction is not necessary after LT and quality of life improves in survivors. Murine models of TT1 suggest that gene therapy or hepatocyte transplantation may have promise for the treatment of TT1 in the future [101,102].

Glycogen Storage Diseases

Hepatic glycogen storage diseases (GSD) are an uncommon group of inherited enzyme deficiency diseases which affect the metabolism of glycogen to glucose. Excess glycogen accumulates in the liver, cardiac and skeletal muscle, kidney, intestines and brain. Diagnosis is based on clinical features and demonstration of specific enzyme deficiency. Types I, III and IV, which are inherited in an autosomal recessive pattern, are associated with significant liver disease.

TYPE I (von Gierke's disease)

Glucose-6-phosphatase (G-6-P) is a hepatic microsomal enzyme, also expressed in the renal tubular epithelium, intestinal mucosa and pancreas. Mutation(s) in the G-6-P gene result in deficiency of the enzyme and glycogen accumulation in the above organs. Affected individuals are dependent on continuous exogenous carbohydrate and infants usually present with fasting hypoglycemia, failure to thrive and growth retardation, lactic acidosis, hyperlipidemia, hyperuricemia and hypoglycemic seizures.

Glycogen overload causes hepatomegaly, hyperbilirubinemia and mildly elevated serum aminotransferase levels. Liver biopsy reveals glycogen accumulation and steatosis but no fibrosis. Histochemical stains for G-6-P are negative and the enzyme is not detectable in the liver. Hepatic adenomas with potential for malignant transformation, are common (up to 50%), especially in children surviving beyond the first decade. Osteoporosis, renal dysfunction and renal calculi are late complications.

Medical management consists of frequent daytime feeding, continuous nocturnal enteral glucose feeds, and use of oral uncooked starch which releases slowly in the intestines. Normal to near normal growth and development can be achieved despite hepatomegaly, dyslipidemia and other abnormalities.

LT is indicated for patients not responsive to medical therapy, and those with progressive liver disease or hepatic masses [103,104,109]. Several reports of LT for GSD I report excellent prognosis with correction of the underlying metabolic defect. Metabolic parameters, hepatic glycogen stores, and patient growth all improve after LT [105-109]. Two reports of combined liver and kidney transplants for GSD I also report good post transplant outcomes [110,111]. Liu et al reported four children with GSD I and one with GSD III who underwent living related liver transplant (LDLT) after which hypoglycemia, hyperlipidemia and acidosis resolved, liver function tests normalized and biochemical abnormalities improved dramatically. Renal function remained normal and all five patients were stable during follow-up periods ranging from 2.2 to 5.5 years [112]. Although short term outcomes post LT appear uniformly good, the long term may be complicated by chronic rejection and immune suppression related nephropathy [104]. Muraca et al report hepatocyte transplantation via a portal-vein catheter in a 47-year-old woman with GSD I induced severe hypoglycemia; 9 months after transplantation, hypoglycemia resolved and the patient was on a normal diet. Thus, hepatocyte transplantation may be an alternative to LT in the future for patients with GSD I without HCC [113].

TYPE III

GSD III results in abnormally structured glycogen due to amylo-1-6-glucosidase (debrancher enzyme) deficiency. The metabolic defect is milder because other mechanisms of gluconeogenesis are functional and the kidneys are spared. In contrast to GSD I, hepatic fibrosis and cirrhosis and skeletal- and cardio-myopathy may develop in the long term. Clinical manifestations and medical management are similar to GSD I except for the need for increased dietary protein intake to provide amino acids for gluconeogenesis. The few reports of LT for GSD III have described good post LT outcomes [103,112].

TYPE IV (Andersen's Disease)

GSD IV is a rare condition caused by a deficiency of the branching enzyme α-1,4-α-1,6-glucosyltransferase, leading to accumulation of amylopectin-like, abnormally shaped diastase resistant glycogen [114].

Clinical manifestations of hepatosplenomegaly, cirrhosis, and death from hepatic failure are seen early in childhood and LT may be necessary within the first 5 years of life. Extra-hepatic accumulation of abnormal glycogen may lead to cardiomyopathy and neuromuscular

disease. Indications for LT include progressive or decompensated liver disease and acute liver failure. LT reverses hepatic disease but extra-hepatic disease may continue to progress.

Fatal cardiomyopathy 9 months after LT was reported in one patient; postmortem evaluations of the heart and brain revealed significant amylopectin accumulation, suggesting progressive extra-hepatic disease despite LT [115]. In another series, [106] two of the seven patients who underwent transplantation for GSD IV died, one from bowel perforation and the other from hepatic artery thrombosis. The remaining five survivors (71%) were stable after 16 to 73 month follow-up periods. One patient showed decreased endomyocardial amylopectin; none of the surviving patients had further cardiac or neuromuscular complications. In contrast, 4 of 13 GSD type IV patients treated with LT because of progressive liver cirrhosis and liver failure, died. Most of the patients (12/13) developed neuromuscular or cardiac complications during follow-up [103]. Collectively, these data suggest that LT benefits GSD IV but there is a significant risk of delayed extra-hepatic complications. Therefore, candidates for LT must be selected with careful pre-transplant cardiac evaluation.

Galactosemia

Galactosemia is a rare (1:40,000 live births) autosomal recessive disorder secondary to deficiency of galactose-1-phosphate uridyl transferase (GALT). Following the initiation of milk feeds in infants with GALT deficiency, galactose and galactose-1-phosphate accumulate in the liver, kidney, lens, and other organs. The disease may manifest in the first few days of life with severe hypoglycemia, encephalopathy, progressive jaundice and liver failure. Cataracts are frequently seen in neonates, and failure to thrive, anemia, gram-negative sepsis, coagulopathy, retarded psychomotor and mental development, hepatomegaly, cirrhosis, and HCC may develop. Learning and growth retardation is more common in girls for unclear reasons; 75% develop ovarian failure [125]. The presence of reducing substances in urine without glycosuria and demonstration of reduced GALT activity in red blood cells confirms the diagnosis. Liver biopsy may show steatosis, periportal bile duct proliferation and hepatic fibrosis and cirrhosis, even as early as at birth. Early diagnosis is important since institution of a lifelong galactose-free diet may prevent disease progression. In the absence of cirrhosis, liver function improves on a galactose free diet. LT should be considered in patients with fulminant hepatic failure, HCC or decompensated cirrhosis. LT appeared to be curative with absence of galactosemia following galactose challenge, and no other complications of galactosemia during a 6 month follow-up period [126].

Progressive Familial Intrahepatic Cholestasis (PFIC)

PFIC includes a group of diseases with persistent intra-hepatic cholestatic jaundice, pruritus, hepatomegaly and developmental delay.

Byler's Disease (PFIC Type 1)

First described in 1969 in an Amish family [127]. Byler's disease (BD) is a rare (1:90,000 live births) autosomal recessive syndrome which causes severe intra-hepatic cholestasis progressing to biliary cirrhosis, chronic liver failure and death, usually during the first decade of life. Another group of children with familial cholestasis had normal gamma glutamyl transferase (GGT) and progressed to cirrhosis [128]. The affected gene ATP8B1 codes for a P-type ATPase. In affected patients, bile salt secretion from biliary canaliculi is decreased and bile salt reuptake from the ileum is increased [133]. Clinical features include jaundice, hepatosplenomegaly, growth retardation, and severe pruritus. Serum aminotransferases and alkaline phosphatase are elevated and GGT may be normal or high. Liver biopsy reveals severe cholestasis and fibrosis or cirrhosis.

Ismail et al compared medical therapy (ursodeoxycholic acid) to LT or partial external biliary diversion in 46 children with BD[129]. Medical therapy resulted in clinical and biochemical improvement in only 10% of patients. With comparable success rates of 80% for both the surgical techniques, the authors recommend biliary diversion for those without cirrhosis. Although improvement is noted with biliary diversion, [129,130] LT is the only therapeutic option once cirrhosis has developed.

Torri, et al reported findings in 12 patients with BD who underwent LT [131]. Median age was 1.32 years (range 0-13), and median post transplant follow-up was 670 days. Two patients (16.6%) died despite re-transplantation for portal and caval thrombosis in one patient and primary graft dysfunction in the other. The remaining patients were alive with excellent actuarial patient and graft survivals of 83% at 1 year and 83% at 5 years.

Soubrane et al [132] report 14 LTs for BD with only one post-operative death after re-transplantation for arterial thrombosis. Among the 13 survivors, graft function, growth, and quality of life were good over an average follow-up period of 17 months (range 6-36 months). Recurrent Byler's disease has not been reported post transplantation and overall, survival after LT is excellent.

PFIC Type 2 and PFIC Type 3 have similar clinical features and are managed similar to PFIC Type 1 [133].

Alagille's Syndrome

Alagille's syndrome (AGS) [134] is a rare (1 in 100,000 live births) autosomal dominant, disorder. The affected gene in AGS is *Jagged1* (*JAG1*) on chromosome 20p12; phenotypic expression is highly variable, even within families [135-137]. There is multisystem involvement, characterized by cholestasis and a marked reduction in the number of the interlobular bile ducts, along with cardiac, renal, facial, ocular, cutaneous, pancreatic, skeletal and neuro-developmental abnormalities.

Bile duct paucity, which progresses over time, is considered the most dominant feature of AGS and is seen in 80-85% of patients. Hyperbilirubinemia in neonates may resolve later in childhood, although severe pruritus may develop in infants even in the absence of jaundice. Hepatic synthetic function is usually preserved despite elevated serum aminotransferases,

alkaline phosphatase and GGT; however, 20% of children with AGS develop cirrhosis and hepatic failure. Cardiovascular disease predicts increased mortality [138]; the most common anomaly is pulmonary artery stenosis followed by tetralogy of Fallot and other intracardiac and peripheral vascular lesions.

A characteristic facies, [139] severe hypercholesterolemia and hypertriglyceridemia, resulting in cutaneous xanthomas, renal abnormalities, CNS anomalies including fatal intracranial hemorrhage, ocular abnormalities, skeletal disease with a characteristic finding of sagittal cleft or butterfly vertebrae are all described. Severe growth retardation results from poor nutrition, severe vomiting, recurrent aspiration pneumonia, steatorrhea and fat malabsorption due to pancreatic insufficiency. Diagnosis of AGS requires demonstration of bile duct paucity associated with at least three of five major criteria: cholestasis, characteristic facies, cardiac anomalies, vertebral anomalies, ocular anomalies. In the first 6 months of age, when ductopenia may be absent, three or four clinical features are sufficient to make the diagnosis. Testing for *JAG1* mutations can be performed in probands and family members.

Treatment consists of maintaining adequate nutrition including medium chain triglycerides and fat soluble vitamin supplementation. Pruritus, the most significant symptom, can be ameliorated with selective use of antihistamines, cholestyramine, rifampin, or ursodeoxycholic acid. Medical therapy and external biliary diversion may help relieve symptoms and postpone LT [140].

LT is indicated in AGS for end stage liver disease, portal hypertension, and severe intractable pruritus and disabling complications prior to development of hepatic failure. LT is associated with higher perioperative risks in patients with AGS, in part due to coexistent severe cardiovascular anomalies. Preoperative cardiac management may improve outcomes [141-143]. Potential donors for living related transplantation should be screened thoroughly because of likelihood of subclinical AGS in relatives.

Survival following LT has varied from 57% to 100% and in long term follow-up up to 9 years, no evidence of recurrent liver disease following LT was seen [141-143]. Cardona, et al [141] reported LT in 12 patients for AGS with all 11 survivors leading normal lives during follow-up between 14 months and 5 1/2 years post LT. Pruritus and xanthomas resolved and skeletal growth improved. Tzakis AG, et al [142] reported LT in 23 children with AGS; 13 (57%) of the children survived between 2-9 years post LT with normal liver function. Three of the fatalities were due severe comorbid cardiovascular disease. Recently, Maldini, et al [143] reported post LT outcomes in 21 AGS patients with a median age 1.95 years (range, 0.7-16.7) at transplantation. With a median follow-up period of 919 days, 18 recipients survived post LT with an actuarial survival rate of 90% at 1 year and 80% at 5 years.

Neonatal Hemochromatosis

Neonatal hemochromatosis (NH) is a rare, severe non-*HFE* related disorder characterized by hepatic and extra-hepatic siderosis, manifesting within the first few days of life. NH may present as acute liver failure. The etiology is unknown although infection, genetic-metabolic

disease, toxic insults and possible gestational allo-immune disease have all been proposed as contributing to NH [87,116,119].

Iron overload can be identified by demonstration of elevated serum ferritin and transferrin-iron saturation (TS). Demonstration of high tissue iron by magnetic resonance imaging or histologic evidence of siderosis in salivary glands can be confirmatory. Postmortem examination reveals hepatocellular collapse, extensive hepatic fibrosis, and siderosis in the liver, heart, kidney, pancreas, and thyroid.

Therapy with antioxidants (vitamin E, N-acetylcysteine, selenium, prostaglandin E1, and desferrioxamine) may temporize the disease course [117]. In a report of 14 infants treated with an antioxidant "cocktail", 5 survived to transplantation and 3 were alive 1 year post transplantation [118].

Medical therapy is not curative and urgent LT appears to be the only definitive treatment. Although the post LT survival is not as favorable as with other diagnoses, [19, 87] this form of therapy may be life-saving [120-123]. Transplantation results in a gradual reduction of systemic iron overload. In one case, there was no re-accumulation of hepatic iron and serial biopsies of buccal mucosa revealed reduction of excess peripheral siderosis over a 5 month follow-up period [123]. However, in another case, iron accumulation was noted in the allograft 7 days after LT and the infant died of cardiac arrhythmias on postoperative day 62 [124]. Autopsy showed hepatic and extra-hepatic siderosis and the rapid iron overloading of the graft was thought to be due to redistribution of excess body iron.

I B. Primary Metabolic Defect is in the Liver: Liver Transplantation Primarily for Extrahepatic Complications

Primary Hyperoxaluria Type I

The primary hyperoxalurias (PHs) are rare autosomal recessive disorders in which deficiency of hepatic alanine: glyoxylate aminotransferase (AGT) (PH type I) or glyoxylate reductase/hydroxypyruvate reductase (GRHPR) (PH type II) results in excess oxalate production by the liver. Excess oxalate is excreted by the kidneys, leading to high urinary oxalate concentrations, calcium oxalate nephrolithiasis and nephrocalcinosis, recurrent urinary tract infections and, if untreated, renal failure in late childhood to early adulthood. Once renal function reduces to less than 50% of normal, plasma oxalate concentration rises and progressive systemic oxalosis may occur, with oxalate deposition in skeletal and cardiac muscle, cardiac conduction system, bone, arteries, ocular and nervous tissue, causing significant morbidity and death. Genetic and phenotypic heterogeneity is noted and diagnosis and therapy must be established early to prevent complications [144-147]. Supportive medical treatment consists of high fluid and low calcium and oxalate intake, supplemented by pyridoxine, and citrate, orthophosphate or magnesium oxide. Hemodialysis may not be adequate to remove overwhelming oxalate production [147,148].

Once the diagnosis of PH is established, LT should be considered to prevent significant renal dysfunction. Now considered the definitive treatment for end stage renal failure in PH type I, combined liver–kidney transplantation replaces the deficient enzyme, correcting the underlying defect and hence preventing failure of the transplanted kidney [147,152,153]. Jamieson [153] recently reported long term, multi-center results from the European PH1 transplant registry. 127 liver transplants were performed in 117 PH type I patients between 1984 and 2004; 75 transplants were either whole or reduced LTs with simultaneous or delayed kidney grafts, 25 were whole or reduced LTs without kidney transplants, and 10 of the 127 LTs were retransplants. The mean age at which a diagnosis was made was 8.8 +/- 9.5 years, the duration on dialysis was 3.2 +/- 3.2 years (range 0-14.4 years), and transplantation was performed at 16.5 +/- 11.4 years. One-, 5- and 10- year patient survival rates were 86%, 80% and 69%, respectively, and, liver graft survival rates were 80%, 72% and 60%. Millan et al, [152] reported 100% patient and graft survival in 6 infants with PH type I who underwent simultaneous liver-kidney transplantation. Mean age at diagnosis was at 5.2+/-3.3 months, mean follow-up period was 6.4+/-1.7 years. Stable long-term kidney allograft function was reported in all patients; skeletal growth and neuro-developmental scores improved after transplantation. Following LT, high urine output must be maintained and renal function must be monitored closely due to mobilization of systemic oxalosis and high renal oxalate load. With oxalate mobilization, major improvement is seen in oxalate loaded tissues including skeletal and cardiac muscle, bone, skin and kidneys [150]. In a mouse model of PH type I, hepatocyte transplantation after hepatic irradiation resulted in decrease in hyperoxaluria and thus may be a potential therapeutic mode in humans in the future [154].

Familial Homozygous Hypercholesterolemia

Familial homozygous hypercholesterolemia is an autosomal recessive disease caused by a deficiency or reduction in the expression of low-density lipoprotein receptors due to a mutant low-density lipoprotein (LDL) receptor gene on chromosome 19. LDL receptors are expressed predominantly (50-75%) in the liver. Hypercholesterolemia, cutaneous xanthomata and cerebrovascular and ischemic heart disease ensue in childhood or adolescence. With severe deficiency (less than 2% of normal LDL receptor activity), cardiovascular death occurs within the first decade of life. In less severe cases (2% to 30% of normal LDL receptor activity), fatal cardiovascular complications develop in adolescence to the third decade of life. Hypercholesterolemia must be treated with a low-fat diet, statin drugs, cholestyramine, nicotinic acid, benzafibrate and LDL aphersis and ileal bypass in some cases. In selected patients, LT may be undertaken to preempt advanced atherosclerosis [159,160,162,166]. LT prior to development of cardiovascular complications replaces a majority of the LDL receptors, decreases plasma cholesterol, may significantly clear xanthomas and may prevent cardiovascular morbidity and mortality. Shotri et al [166], report long term results in 4 patients after LT for familial hypercholesterolemia. Two patients remained well 11 years and 4 years post LT, one patient had a fatal myocardial infarction 2 years after LT and a third patient required 3 LTs but was alive 12 years later. Serum cholesterol normalized in all patients. There are rare reports of LT with simultaneous coronary artery bypass grafting

[163], or shortly after heart transplantation [164]. If advanced heart disease is noted, a combined heart-LT is indicated as the best solution to correct the underlying defect and prevent morbidity in the heart graft [165].

Criggler-Najjar Syndrome Type I

Crigler-Najjar (CN-I) syndrome type I is an autosomal recessive disorder due to an absence of bilirubin uridine-diphosphate glucoronyl transferase that results in severe unconjugated hyperbilirubinemia in the neonate.

Soon after birth, exchange transfusions for 12-16 hours/day followed by phototherapy are acceptable in infants. This transforms un-conjugated bilirubin into water-soluble fragments and is efficacious in resolving jaundice. Over time and with older children, such treatment is less acceptable because of its impact on lifestyle and is less effective. Children are physically and mentally normal until they develop kernicterus, which can precipitate without warning and cause irreversible neurologic damage. Van der Veere et al [156] reported results of a world registry of 57 patients with Crigler-Najjar syndrome type I. 21 patients received liver transplants at a mean age of 9.1 years. Five of eight patients with significant preoperative neurologic disease had no significant neurologic improvement after transplantation. LT corrects the underlying metabolic defect, is curative with no recurrence after transplantation, and in a jaundiced but otherwise healthy child with CN-I, must be undertaken to preempt the development of irreversible neurologic damage [84,155-157]. Auxiliary LT is possible and may have the added utility of sparing the native liver for the potential of future gene therapy or hepatocyte transplantation [158].

Urea Cycle Defects

Ornithine Transcarbamylase Deficiency

Deficiencies of urea cycle enzymes may lead to severe, fatal, hyperammonemic encephalopathy. Several enzyme deficiencies have been characterized; ornithine transcarbamylase (OT) deficiency is the most common of these, and is discussed here as a representative disorder. Being X-linked (gene locus Xp21), it is a semi dominant disease with variable phenotypic expression and affects both males and females. OT is a mitochondrial enzyme, operative in the synthesis of citrulline from ornithine and carbamyl-phosphate and hence in the detoxification of ammonia. It is predominantly (80%) active in the liver and is also present in intestinal mucosa.

In the hemizygous male [167] OT deficiency results in profound elevation in ammonia and glutamine, and depletion of arginine and citrulline. Severe hyperammonemia in the neonate is a common presentation and causes coma and irreversible brain injury and can be fatal unless promptly and aggressively treated. Those who survive the neonatal period and those with late onset of symptoms may suffer mental retardation, cerebral palsy and seizures. The clinical presentation is variable among heterozygous OT deficient females with clinical symptoms presenting in the first two years of life or around puberty. However, even females

with mild disease initially are at high risk for irreversible neurologic damage; 15% develop severe hyperammonemia and may have severe mental retardation [168-170].

Initial treatment in females and supportive treatment in males consists of a low-protein diet supplemented with essential amino acids and either sodium benzoate or sodium phenylbutyrate to achieve a net deficit of nitrogenous waste by decreasing urea synthesis and increasing nitrogen-waste excretion. However, in one review of medical therapy for OT deficiency in 32 females (age 1- to 17-years) 23 patients had at least 1 hospitalization for hyperammonemia and 16% had intellectual decline during therapy [168]. Another report described fatal postpartum hyperammonemia in a 25-year-old OT deficient woman despite medical therapy into adulthood [170].

LT is the only definitive treatment leading to long-term survival for male OT deficient hemizygotes. In females, the need for LT is dictated by symptom severity and response to medical therapy. The frequency and duration of hyperammonemic episodes impacts on the individual patients' intellectual development and neurologic function [167].

LT for urea cycle enzyme deficiencies is associated with prompt reduction in serum ammonia levels, improvement in plasma amino acid profiles and variable neurologic improvement [171-175]. Whitington et al [175] described the results of LT in 16 patients; 10 were OT deficient, 3 were carbamylphosphate synthase deficient and 3 had citrullinemia. Fourteen (87.5%) patients survived with follow-up ranging from 11 months to 6 years and none required retransplantation. The two deceased patients were twin boys with OT deficiency and severe neurologic impairment. One death occurred within a week after LT and the other twin with profound brain damage succumbed to pneumonia 9 months after LT. Ammonia levels became normal in all survivors. Post LT, almost no developmental improvement was seen in 6 moderately impaired children; 4 mildly impaired children were functional with mild disabilities and 4 patients who were normal pre-LT had normal development and function. LT should therefore be performed in patients with hyperammonemic urea cycle defects prior to the onset of neurologic dysfunction.

Hemophilia

Hemophilia A and B are X-linked recessive disorders and are caused by deficiency of coagulant factor VIII and factor IX respectively. Clinical manifestations include spontaneous bleeding, especially into joints and soft tissues causing significant morbidity. Pooled factor concentrate infusions although life saving, have been complicated by infection with hepatitis B and C, and HIV. Recombinant factors VII and IX are now available and gene therapy is being explored [176,177]. Although LT in hemophiliacs is usually performed for end-stage liver disease due to HBV or HCV, the underlying condition is also cured because of normal coagulation factor synthesis by the donor liver [179-183]. Factor VIII or factor IX levels increase dramatically within the first day post-transplant and remain normal over extended follow-up [179]. With peri-operative coagulant factor replacement, LT can be safely performed without significant bleeding complications [178-183]. Wilde, et al reported outcomes of LT in hemophiliacs with chronic hepatitis C infection [183]. Eleven patients underwent LT with supplemental factor concentrate infusion. Nine patients were alive a

median of 5 years post LT (6 months to 11 years). One patient died 6 years post LT due to a myocardial infarction and the other died of liver failure from recurrent hepatitis C. The outcome of LT in hemophiliacs is good and is associated with relatively little morbidity.

Amyloidosis

Amyloidosis is an uncommon disease resulting from tissue deposits of an insoluble fibrillar protein. The diagnosis can be confirmed by demonstration of apple-green birefringence on polarization microscopy after Congo red staining. Deposition of amyloid material in various tissues can lead to variable disease expression [192-194]. Classification is based on identification of the precursor protein; the incidence, management options and prognosis depend on the underlying etiology [192-194].

Familial Amyloidotic Polyneuropathy

Familial Amyloidotic Polyneuropathy (FAP) is a hereditary amyloidosis due to accumulation of one of the FAP-related mutated proteins: amyloidogenic transthyretin (ATTR), apolipoprotein A-I or gelsolin. Worldwide, FAP due to mutated transthyretin (ATTR-FAP) is the most common. Transthyretin (TTR) is a protein of unknown function, predominantly synthesized in the liver. It circulates in the blood stream bound to thyroxine or to a retinol binding protein-Vitamin A complex. The *TTR* gene locus is 18q12.1 and mutations in *TTR* results in an abnormally folding transthyretin which deposits systemically as amyloid fibrils [77,79]. ATTR-FAP due to a Val30Met mutation in transthyretin was first described by Andrade in 1952 [78]. Since then more than 100 different mutations in the *TTR* gene were identified, with a worldwide distribution [79].

ATTR-FAP is an autosomal dominant disorder with varied clinical manifestations, even among patients with the same mutation in the *TTR* gene. ATTR-FAP is classified into neuropathic, oculoleptomeningeal and cardiac forms based on clinical phenotype. Clinical manifestations, thought to be due to deposition of abnormal amyloid fibrils, are protean and include sensorimotor and autonomic polyneuropathy, ocular amyloidosis, cerebral amyloid angiopathy, gastrointestinal dysmotility, cardiac dysfunction and arrythmias, renal dysfunction and anemia [77]. In autopsy findings [64], amyloid fibril deposits in the nervous system were predominant in the peripheral nerves, anterior and posterior roots of the spinal cord, spinal ganglia, autonomic nervous system and the choroid plexus. Decrease in myelinated and unmyelinated nerve fibers was associated with degenerative changes in Schwann cells. Amyloid deposits were also frequent in the cardiovascular system and the cardiac conduction system, the gastrointestinal system, the kidneys and the thyroid. Diagnosis is established based on clinical and pathologic findings and confirmed by genetic testing.

FAP disease manifestations are progressive, disabling and fatal if untreated. Because ATTR is predominantly synthesized in the liver, LT for ATTR-FAP eliminates ATTR production and is presently the only known curative treatment [35,63]. Since disease manifestations are mostly irreversible and may advance rapidly, LT is recommended at the onset of first symptoms and before involvement of neurologic, cardiac, renal or

gastrointestinal systems. Several recent series reported good outcomes post LT [35,207,208,211]. Adams, et al [207] report overall 1- and 5- years survival rates of 82% and 60% respectively. Recent data [35] from the Familial Amyloidotic Polyneuropathy World Transplant Registry report 579 LTs for 539 patients with FAP between 1991-2000 and 60 LTs for FAP in 2003. An excellent overall 5-year survival of 77% was observed which is comparable to post-LT survival for other chronic liver disorders. Among the deceased, death occurred mainly from cardiac complications (39%) [35]. Post-LT, circulating mutant transthyretin is absent and neuropathy improves [206-208,211].

Because the manifestations of FAP occur late and the FAP liver is morphologically and functionally normal, the explant FAP liver is thought to be appropriate for sequential transplantation into a non-FAP patient with end stage liver disease [30,35,209,210]. There was no increase in risk to the donors of FAP livers when compared to patients with FAP whose livers were not used for transplantation. Although mutant TTR was present in circulation and FAP manifestations may possibly develop in the recipient of an FAP liver, symptoms of FAP were not reported to this point [30,35,209,210].

Hereditary Protein C Deficiency

Protein C (PC) is a vitamin K-dependent plasma protein that prevents formation of blood clots by degrading activated factors Va and VIIIa in the coagulation cascade when activated by a thrombin-thrombomodulin complex on capillary endothelium [229]. A deficiency in protein C thus results in a hypercoagulable state. PC deficiency may be acquired or inherited as an autosomal dominant disorder; homozygous, heterozygous and compound heterozygous states have been described. The penetrance is variable. Homozygous PC deficiency usually presents in the neonatal period as severe fatal purpura fulminans, disseminated intravascular coagulation (DIC) and thromboembolism. Heterozygotes may present with purpura fulminans, venous thrombosis and/or pulmonary embolism. Inherited PC deficiency may present later in adulthood. Protein C concentrates or fresh frozen plasma are used for acute and short term treatment in deficient states but are impractical in the long term, in part because of the short half life of protein C [230]. Warfarin, heparin or low molecular weight heparins are also effective to prevent thrombosis but carry a risk of bleeding [230]. When medical treatments fails, LT is indicated for severely symptomatic homozygous protein C deficiency [231,232] LT results in normalization of protein C levels and reverses the hypercoaguble state [231].

II A. Primary Defect is Extrahepatic: Liver Disease May Recur After Transplantation

Hereditary Hemochromatosis

Hemochromatosis is a disease that results from excessive deposition of iron in various tissues, particularly in the liver. Cellular toxicity from excess iron leads to structural damage

and functional insufficiency. The Online Mendelian Inheritance database classifies Primary Iron-Overload disorders into four subtypes [37,38]. Classic Hereditary Hemochromatosis (HH) or "Type 1" or *HFE* related hemochromatosis is the most common form. Clinical disease generally presents in middle age and phenotypic expression is milder in women compared to men. Other types of Hereditary Hemochromatosis include "Type 2" or Juvenile Hereditary Hemochromatosis. This form of HH is characterized by severe phenotypic expression in the second and third decade of life; men and women are affected equally. Type 2 HH is associated with mutations in Hemojuvelin or Hepcidin; "Type 3" HH is associated with mutations in Transferrin receptor 2. "Type 4" HH form of inherited iron overload has also been called "Ferroportin disease". The following section will focus on the features, diagnosis, management and LT in classical or *HFE*-associated HH. HH was identified as an inherited, autosomal recessive disorder closely linked to the Major Histocompatibility complex HLA-A3 locus [40]. Feder, JN et al [41] reported the discovery of the candidate hemochromatosis '*HFE*' gene in 1996. HH is among the most common inherited disorders. It is estimated that the phenotypic prevalence is 1:400 and genotypic prevalence is 1:200 in individuals of northern European ancestry [42]. *HFE* related hemochromatosis is characterized by a homozygous substitution of cysteine to tyrosine at residue 282 of the *HFE* protein (C282Y). Over 85% of those with phenotypic HH are homozygous for C282Y. A second mutation H63D in the *HFE* gene may increase disease susceptibility in compound heterozygotes (C282Y/H63D).

The clinical presentation of HH is variable ranging from an incidental diagnosis, to weakness and lethargy, arthralgias and arthritis, or advanced disease characterized by diabetes mellitus, liver disease, hypogonadism, cardiac disease (heart failure, arrhythmias), hypothyroidism [42]. Chronic liver disease with or without cirrhosis is common and decreased life expectancy and HCC are associated with cirrhosis. The diagnosis and management of HH, which has been reviewed recently [39,42,43] is based on history and physical examination, family history and supported by laboratory, imaging and HFE gene testing. The initial screening test is a serum transferrin-iron saturation (TS); if TS is >45%, a repeat fasting measurement should be done along with a serum ferritin level. This is followed by *HFE* gene testing and in selected patients, with liver biopsy. It is important to recognize that in the setting of end-stage liver disease, serum TS may frequently be elevated in the absence of *HFE*-hemochromatosis. Furthermore, the majority of patients with end-stage liver disease who have otherwise unexplained marked hepatic iron overload do not have *HFE*-hemochromatosis [58,59,149]. In addition, liver biopsy is not optimal in patients with advanced cirrhosis due to increased risk of complications and the marked heterogeneity in iron deposition within the liver in this setting may lead to sampling variability in tissue iron measurement [88]. New methods for non-invasive measurement of hepatic iron content are being developed, such as MRI and susceptometry and may be much more helpful in cirrhotic patients being evaluated for HH.

The mainstay of medical treatment is weekly phlebotomy of 500 ml of blood (0.25g iron) until serum ferritin is $\leq$ 50µg/L. Maintenance therapy after adequate iron depletion generally requires approximately 4-12 phlebotomies per year. Early diagnosis and phlebotomy therapy prior to development of cirrhosis is associated with normal life expectancy. Despite the high frequency of *HFE* mutations, advanced liver disease requiring transplantation for *HFE*-

associated HH is unusual [45,46,50]. However, if cirrhosis is present at the time of diagnosis, despite adequate phlebotomy therapy, life expectancy is diminished and there remains an increased risk of HCC [44,48]. LT is the only effective treatment for patients with decompensated cirrhosis secondary to HH and those found to have small HCC [19]. Iron depletion via phlebotomy may improve post transplantation survival and must be attempted in these patients before transplantation. Patients undergoing LT for *HFE*-HH have a significantly decreased survival compared to patients with other diagnoses [48-53]. Furthermore, some studies suggest that significant hepatic iron overload from any cause is associated with decreased survival after transplantation [49,51,54,55].

Prior to identification of the *HFE* gene, several studies reported results of LT for hemochromatosis. Pillay et al initially reported a 83% post LT survival for greater than six months in patients with end stage HH liver disease after iron depletion therapy [56]. A subsequent analysis of LT data reported to Medicare revealed a 54% 1 year survival an 43% 5 year survival after LT for HH, both significantly lower than LT for other indications. This study included pediatric patients and patients with other causes of iron overload in the hemochromatosis group [50]. This was similar to findings from the Pitt-UNOS Liver Transplant Registry where 1- and 5- year survivals of 65% and 56% were noted after LT for HH [12]. Kowdley et al [48] reported results of LT for a presumed diagnosis of hereditary hemochromatosis in 37 patients from 5 LT centers. This cohort had a higher than expected prevalence of HCC, and 1- and 5-year survivals were similar to that reported by Kilpe et al [50]. More recently, Brandhagen et al [49] compared long term patient and graft survival and LT complications in 41 patients who had iron overload evidenced by a hepatic iron index of greater than 1.9 with 41 matched LT recipients without increased hepatic iron. Post LT 5-year patient survival was significantly lower in cases with hepatic iron overload compared to matched controls without iron excess (48% vs. 77%; P = .045). The reduced survival was attributable mainly to fatal infections in patients with iron overload (24% vs. 7%; P = .03). Notably however, only 4 of the 41 patients with iron overload were C282Y homozygotes. Other studies [54,57] also suggest that most patients with hepatic iron overload in the setting of end-stage cirrhosis do not have *HFE*-HH.

Although hepatic iron overload in the setting of end-stage liver disease appears most common among patients with chronic hepatitis C and alcoholic liver disease, it has also been described among patients with other types of end-stage liver disease [58,59]. Some authors have suggested that hepatic iron overload is associated with a poor outcome after LT regardless of the presence or absence of *HFE* mutations [54,59]. A recent study assessed post LT outcomes in 22 patients with HH. HH patients had relatively poor outcomes following transplantation with 1-, 3-, and 5- year survivals of 72%, 62% and 55% respectively [60].

In a recent large multi-center study, Kowdley et al examined the prevalence of *HFE* mutations in liver transplant recipients with known or suspected hepatic iron overload and the relationship between *HFE* genotype and survival after LT [53]. *HFE*-HH was noted in 12.8% of patients of whom 14 were C282Y homozygotes (7.2%) and 11 were C282Y/H63D compound heterozygotes (5.6%). Post LT survival was significantly lower among patients with HH (1-, 3-, and 5-year survival rates of 64%, 48%, 34%, respectively) compared with other genotypes. After adjustment for age, United Network for Organ Sharing (UNOS) status, year of transplantation, and either elevated hepatic iron index or hepatic iron concentration,

patients with HH had a hazard ratio for death of 2.6 (P = .002). Non-HH patients with hepatic iron overload also had significantly decreased survival when compared with those without hepatic iron overload.

The available data suggest that in patients with hepatic iron overload, post-LT short term mortality is predominantly due to bacterial, and fungal infections and cardiac complications, while long term survival is affected by recurrent HCC and cholangiocarcinoma or cardiac complications [48,51,53,60,61,199].

A few studies which examined iron re-accumulation in the transplanted liver in patients with iron overload have found conflicting results. Some studies reported no significant iron loading in the transplanted liver and others the opposite [60]. It is also not clear whether iron accumulation or mobilization occurs after transplantation of an *HFE*-HH liver into a wild-type individual [60].

Gaucher Disease

Gaucher disease (GD) is a rare autosomal recessive disorder resulting in deficiency of the enzyme glucocerebrosidase in leukocytes, bone marrow, hepatocytes and aminocytes. It is highly prevalent in Ashkenazi Jews and the most common of lysosomal storage disorders. It is inherited in an autosomal recessive pattern, and results in accumulation of glucosylceramide within lysosomes of reticuloendothelial cells. The majority of patients (99%) have non-neuronopathic disease (type 1), which is characterized by hepatosplenomegaly, pulmonary disease, pancytopenia and osteolytic lesions due to infiltration by Gaucher's cells. Hematologic and other malignancies including rare instances of HCC [187] have been reported. GD may rarely present in infancy with acute liver failure but more commonly presents in late childhood with the above manifestations. Brain involvement is seen in GD types 2 and 3. Although hepatocytes are not part of the storage disorder, the liver is universally involved in GD type 1 and elevated aminotransferases are a common feature. Uncommonly, liver failure, cirrhosis, portal hypertension and severe hepatic fibrosis are reported [184-186]. Diagnosis is made by demonstrating large multinucleated Gaucher's cells in marrow aspirate and confirmed by enzyme assay. Gaucher's cells are also found around hepatic central veins obstructing the sinusoids.

In the few reported cases of LT [189-191] for hepatic complications of GD type I, liver function improved, but, glucocerebrosidase activity may not return to normal [191], and Gaucher cells may re-accumulate in the liver [190]. One patient with GD type 1 who underwent LT for HCC sustained remission of her Gaucher's disease and was medically stable without enzyme supplementation 2 1/2 years post LT [186]. With over 4,300 people currently under treatment, the standard of care for GD type 1 is enzyme replacement therapy with macrophage-targeted recombinant glucocerebrosidase (Imiglucerase) [188].

Erythropoietic Protoporphyria

The porphyrias are a varied and uncommon group of genetic or acquired disorders due to defects of enzymes in the heme biosynthesis pathway. Erythropoietic or Erythrohepatic Protoporphyria (EPP) is caused by deficiency of the enzyme ferrochelatase which catalyzes the terminal step of heme synthesis. [47] EPP is an autosomal dominant disorder with variable penetrance even within individual families. Photosensitivity is the predominant manifestation of EPP. Accumulation of protoporphyrin in skin and dermal blood vessels leads to acute cutaneous erythema with burning and itching. If sun exposure is prolonged, formation of bullae and vesicles and increased skin fragility resulting from mild trauma to light exposed skin is noted. EPP patients must be monitored for liver disease and those with erythrocyte protoporphyrin levels over 1500mcg/dL and elevated liver enzymes must be considered for liver biopsy. Optimal medical therapy is not well defined and may include erythrocyte transfusion, intravenous heme, high dose beta carotene, oral charcoal, oral cholestyramine or oral chenodeoxycholic acid.

In approximately 10% of patients with EPP, protoporphyrin accumulation may lead to chemical liver injury and severe liver disease. When excess protoporphyrin produced in the bone marrow exceeds the limit of biliary canalicular excretion, protoporphyrin accumulates in the liver resulting in local toxicity, cholestasis, nodular cirrhosis and pigmented gallstones associated with hemolysis. Progressive liver disease, cirrhosis, and hepatic failure may develop, necessitating LT [68,86]. Several reports of LT for EPP have shown favorable prognosis [67,68,86,178].

Ferrochelatase deficiency is unchanged and excess protoporphyrin production in the bone marrow continues after LT. Protoporphyrin levels in erythrocytes and in feces remain elevated after LT [67,68,178]. Recurrent, progressive liver injury is noted after LT and is treated as discussed above and selected patients may be considered for retransplantation when indicated [68,202]. Neurologic dysfunction and hemolysis may complicate the perioperative period due to high serum protoporphyrin levels and splanchnic bed exposure to operating room lights [86,200]. Preoperative plasmapheresis, erythrocyte transfusion and filters to remove 400-nm wavelength from operating room lights are recommended [201].

Nonalcoholic Steatohepatitis

NonAlcoholic SteatoHepatitis (NASH) is a unique syndrome in which histopathologic findings are similar to alcoholic hepatitis despite an absence of history of alcohol abuse in affected patients [212]. It is commonly associated with metabolic syndrome (obesity, dyslipidemia and insulin resistance/diabetes mellitus type 2) [213,214] or following jejuno-ileal bypass surgery for morbid obesity. The true prevalence of NASH is difficult to determine as the diagnosis is based on liver histology but is believed to range in the United States from 3% in the general population to more than 40% in morbidly obese patients [215,216]. The diagnosis is frequently made in patients referred for evaluation of elevated serum aminotransferases. Symptoms are nonspecific and may include right upper abdominal pain and fatigue. Exclusion of other etiologies for liver disease and presence of characteristic

histology findings on liver biopsy are necessary to establish a diagnosis of NASH. Liver histology may range from macrovesicular steatosis without necroinflammation or fibrosis (NAFLD) to steatosis in addition to ballooning degeneration of hepatocytes, necroinflammation, and variable degrees of fibrosis (NASH) [199].

The pathogenesis, natural history and prognosis of NASH are being actively studied at present. There are several excellent recent reviews providing an overview of NAFLD/NASH [196-198]. Briefly, it has been suggested that insulin resistance is the first step in this process, leading to hepatic steatosis; increased oxidative stress in the liver via a number of possible pathways may subsequently lead to necro-inflammatory changes and fibrosis. At the time of initial biopsy, 30-40% of patients with NASH have advanced hepatic fibrosis and 10-15% have cirrhosis [216,219-221]. Over time, a significant percentage of patients with NASH have progressive disease including worsening cirrhosis and portal hypertension and liver related death in 20% of cirrhotic NASH patients [222]. In late stages of the disease, steatosis may not be apparent on histology leaving a picture of “bland” or undifferentiated cirrhosis (cryptogenic cirrhosis). It is now being recognized that a significant proportion of patients with cryptogenic cirrhosis likely have “burned out” NASH, given that a high proportion of these patients have type 2 diabetes, obesity and other features of the metabolic syndrome [151,195]. Steatosis and NASH has also been described more commonly among patients after LT for cryptogenic cirrhosis compared to other diagnoses [225,226]. An increased prevalence of HCC in patients with NASH is also reported [223-224]. In one study, 6.9% of HCC cases were seen in patients with cryptogenic cirrhosis and clinical features of NASH [223], although in another study there was no progression of NASH cirrhosis to HCC in 23 patients over 5 years [222].

Medical therapy is not curative but may have some benefit in improvement of steatohepatitis. Therapy is aimed at improving insulin sensitivity via weight loss and medications (thiazolidinediones and metformin), decreasing oxidative injury (Vitamin E, Betaine) and treating dyslipidemia (gemfibrozil, atorvastatin).

LT is indicated for and is routinely performed in patients with decompensated cirrhosis attributed to NASH. In one series reported by Charlton et al 2.9% of liver transplants from 1993-1998 at Mayo Clinic were for end stage liver disease due to NASH. This proportion likely underestimates the proportion of patients progressing from NASH to decompensated liver disease requiring LT, given that many are classified as “cryptogenic”. Following LT, a high incidence of recurrence of steatosis and steatohepatitis with progression to cirrhosis and decompensated liver disease is well documented in numerous studies [221,227]. In the series [221] by Charlton et al, recurrent steatosis was noted in 60% of those who underwent LT for NASH in contrast to 15% of those with alcoholic liver disease, 15% of those with hepatitis C and 5% of those with cholestatic liver disease. Steatohepatitis recurred in 33% of transplant recipients with NASH, and progressed to cirrhosis in 12.5% [221].

The recurrence of NASH following LT clearly demonstrates that LT does not cure the metabolic defect in NASH. Predisposing factors such as obesity, diabetes and dyslipidemia must be aggressively treated and in selected cases, gastric bypass or bariatric surgery may be beneficial [228].

II B. Primary Defect is Extrahepatic: Liver Transplantation Curative for Hepatic Component of Generalized Disorder

Cystic Fibrosis

Cystic fibrosis (CF) is a common autosomal recessive disorder, affecting approximately 1 in 3000 live Caucasian births. The genetic defect is a mutant cystic fibrosis transmembrane regulator (*CFTR*) gene on chromosome 7, which codes for a CAMP-dependent chloride channel on the apical membranes of epithelial cells lining the airways, pancreatic ducts, biliary tree, sweat ducts, intestines, and vas deferens. More than 1,000 mutations have been described with varied phenotype and penetrance. Depending on the criteria used to define liver disease, the reported prevalence of CF-associated liver disease (CF-LD) widely ranges from 2 to 68% in children and adolescents. CF-LD generally manifests within the first decade of life with increasing prevalence until the age of 12-14 years. In two recent long term longitudinal studies, approximately 27%-41% of CF patients developed CF-LD [233,234]. 22% of those with CF-LD progressed to cirrhosis [233]. The overall incidence of advanced CF-LD in patients with CF is estimated at 1.9% -7.8% [233,234]. Presentation of advanced CF-LD liver disease is less common beyond 12-14 years of age [233, 234]. Livers from 2 adults with CF without CF-LD, who died from complications post lung transplantation were successfully transplanted with recipients surviving over 4 years [237].

The absence of apical membrane CFTR in bile duct epithelial cells results in obstruction of hepatic bile ductules by abnormal mucoid secretions or inspissated proteinaceous bile [235]. Common bile duct obstruction may result from pancreatic fibrosis or sclerosing cholangitis. The histologic features are characterized by periductular inflammation and focal biliary cirrhosis with preserved liver function, which may progress to mulilobular cirrhosis, followed by hepatosplenomegaly, portal hypertension, ascites, variceal bleeding and liver failure. The presence of portal hypertension indicates a poor prognosis, as the mean survival after diagnosis is 4.5 years [236]. CF-LD correlates with a history of meconium ileus and with pancreatic insufficiency [233,234] and in some studies, with severe *CFTR* mutations. With better outcomes in pulmonary CF, life expectancy has increased and advanced CF-LD is more prevalent and is now considered the second leading cause of death in these patients.

Standard medical therapy in CF-LD consists of high-dose ursodeoxycholic acid to counter effects of cholestasis and nutritional support supplemented by pancreatic enzymes and fat soluble vitamins. LT is the only curative therapy for advanced CF-LD. Significant portal hypertension is associated with progressive decline in pulmonary function[240] and it is important to evaluate CF-LD patients early for LT, before the onset of hepatocellular decompensation [205,238,240,241-245]. Genyk et al [238] suggest a scoring system to identify patients for referral to LT. Transjugular intra-hepatic portosystemic shunt (TIPSS) or portosystemic shunt surgery in children are useful temporizing measures in patients with compensated CF cirrhosis with refractory variceal bleeding or in those with contraindications for LT [242,243].

Although LT for CF-LD was initially feared to be complicated by significant peri- and post-operative pulmonary morbidity and mortality, several studies report very good outcomes [239,244-246]. It is of paramount importance to assess, aggressively treat and stabilize lung function in CF with chest physiotherapy, nebulized bronchodilators, nebulized DNAse, nutritional support and antibiotics when indicated. These measures should be continued post LT. Candidates for isolated LT should have stable lung function with forced vital capacity (FVC) $\geq$ 75% of predicted value, and forced expiratory volume in one second (FEV1) $\geq$ 70%. Although lung colonization with multi-drug resistant bacterial organisms may be a contraindication for isolated LT in many centers, Aspergillus lung colonization, treated with antifungal therapy is not considered an absolute contraindication for LT [203,248].

Combined lung-liver or heart-lung-liver should be considered in patients [217,218,240,241,245,246] with severe pulmonary disease, hypercarbia, frequent pulmonary infection, colonization with multi-drug resistant organisms, extensive pulmonary fibrosis, hepatopulmonary syndrome or severe pulmonary hypertension. A combined lung-liver or heart-lung-liver may result in the liver exerting an immunotolerant effect with a theoretically lower likelihood of rejection of grafted lung or heart. Recipients of combined lung–liver transplants showed a favorable short term prognosis [217,218] while heart–lung–liver triple transplant recipients tend to be older and have a poor short term prognosis [218,248].

Following LT, prognosis is comparable to LT for other indications and appears to be better in pediatric patients [247] compared to adults. Overall, a long-term survival of 75% has been reported with improvement of lung function, quality of life, nutritional status and bone mineralization [204,205,239,240,244-246,247]. Short term and long term morbidity and mortality in patients with CF-LD after LT is predominantly related to pulmonary infections, sepsis and worsening lung function [203-206,238,240-247]. However, overall lung function improves likely due to a combination of improved nutrition, improved lung mechanics due to resolution of ascites and correction of hepatopulmonary syndrome induced hypoxemia [239,240,244,246,247,248]. Furthermore, it is possible that airway inflammation may be ameliorated by immunosuppressive medications.

A higher incidence of biliary complications is noted in CF-LD patients with duct-to-duct biliary anastomosis during LT; therefore Roux-en-Y choledocho-jejunostomy is preferred for biliary reconstruction [245]. However, a Roux-en-Y choledocho-jejunostomy may be associated with malabsorption because of a shorter bowel length. The standard immunouppressive regimen following LT includes anti-IL-2 monoclonal antibody, tacrolimus (FK506), and prednisone. In those with uncontrolled or worsening diabetes mellitus microemulsified cyclosporine may be substituted for tacrolimus.

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